Masthead Logo

University of Connecticut
OpenCommons@UConn

Master's Theses

University of Connecticut Graduate School

5-12-2019

The Role of AKAP18 and Protein Phosphatase Inhibitor-1 in the Heart: Defining their Binding Domain

Shania Aponte Paris
aponteparis@uchc.edu

Recommended Citation
Aponte Paris, Shania, "The Role of AKAP18 and Protein Phosphatase Inhibitor-1 in the Heart: Defining their Binding Domain" (2019). *Master's Theses*. 1375.
https://opencommons.uconn.edu/gs_theses/1375

This work is brought to you for free and open access by the University of Connecticut Graduate School at OpenCommons@UConn. It has been accepted for inclusion in Master's Theses by an authorized administrator of OpenCommons@UConn. For more information, please contact opencommons@uconn.edu.

The Role of AKAP18 and Protein Phosphatase Inhibitor-1 in the Heart: Defining their Binding Domain

Shania N. Aponte París

B.S., Antillean Adventist University, 2016

A Thesis

Submitted in Partial Fulfillment of the

Requirements for the Degree of Master of Science

At the

University of Connecticut

2019

© Copyright by

Shania N. Aponte París

All Rights Reserved

2019

APPROVAL PAGE

Masters of Science Thesis

The Role of AKAP18 and Protein Phosphatase Inhibitor-1 in the Heart: Defining their Binding Domain

Presented by

Shania N. Aponte París, B.S.

Major Advisor_____
Kimberly Dodge-Kafka

Associate Advisor _____
Lixia Yue

Associate Advisor_____
Anne Delany

University of Connecticut

2019

ACKNOWLEDGEMENTS

I would first like to thank the Young Innovative Investigator Program (YIIP) for providing me the opportunity and the tools to conduct research at the University of Connecticut. Thank you Dr. Barry, Lana Angelo, Dr. Ruaño and my M1 mentors Dr. Delany, Dr. Zempsky and Dr. Cavallari for your encouragements, advices and your guidinance throughout this amazing journey.

To the Graduate School and the Calhoun Cardiovascular Research Center at UConn Health: thank you for your incredible service and your support these past three years.

I must also express my very profound gratitude to my amazing PI and mentor Dr. Dodge-Kafka. I came into your lab with almost no lab experience and I deeply thank you for taking the time to teach me everything I know. Your door was always open for any questions I may have regarding lab tecniques, thesis, conferences, courses and so forth. Thank you for sitting down with me multiple times to go over my presentations, your inputs helped me become a better presenter. Thank you for guiding me throughout my thesis writing and defense. I am also grateful of my lab members, Moriah Gildart for helping me understand research papers, and also answering any questions regarding protocols and material. I am also thankful of my other lab member Kristin Tokarski and our lab technician Fabrizio Tropea for their support.

I would also like to acknowledge my supplemental grant sponsored by the National Heart, Lung, and Blood Institute which funded me throughout these past two years. As well as my committee

members, Dr. Yue and Dr. Delany, for their guidance and support through my thesis. I am also deeply grateful for Dr. Bezsonova and her lab for helping me with the NMR experiments.

Last but not least, I would like to thank my parents Santos and Sonia, my sisters Stephanie and Shiara, and the rest of my family for their amazing support throughout my academic journey. I am also deeply grateful for the friends I have acquired here in Connecticut, their support and friendship helped me feel less homesick.

To all, thank you for providing the tools, support and mentorship necessary for me to finish my Master's thesis and also be successful in an academic environement.

TABLE OF CONTENTS

COPYRIGHT STATEMENT…….....ii

APPROVAL PAGE……iii

ACKNOWLEDGEMENTS……iv

LIST OF FIGURES……..viii

LIST OF TABLES……..ix

ABBREVIATIONS……x

ABSTRACT……...xi

CARDIOVASCULAR DISEASE……..……1

Figure 1. Cardiovascular disease statistics…….……...….2

Figure 2. Heart failure cycle of events……..*3*

A-KINASE ANCHORING PROTEINS (AKAPS) …….……...…4

Figure 3. β-adrenergic signaling and AKAP features……..*6*

AKAPS IN THE HEART……7

Table 1. Common AKAPs in the heart…….……8

PROTEIN PHOSPHATASE INHIBITOR-1 (I-1)…...…….....9

Figure 4. Schematic illustration of Inhibitor-1 (I-1), with known binding domains and phosphorylation sites……10

AKAP18……..11

Figure 5. AKAP18 spliced variants……...12

Figure 6. Schematic drawing of AKAP18 role in Ca^{2+} cycling…….13

METHODS……14

RESULTS……..17

Figure 7. AKAP/I-1 binding is required for I-1 phosphorylation and I-1 binds to the 1-75 segment of AKAP18γ……..…18

AKAP18/INHIBITOR-1 BINDING DOMAIN……....20

Figure 8. Illustration of protein purification assays and protein stain of AKAP18γ (1-75) and I-1……..21

NMR SPECTROSCOPY RESULTS…….23

Figure 9. HSQC spectrum of the 1-75 segment of AKAP18γ and PSIPRED 3.3 protein structure prediction results of AKAP18γ (1-75).……...24

Figure 10. PSIPRED 3.3 results for I-1 secondary structure prediction……..……25

Figure 11. Two-dimensional HSQC titration experiment……..27

Figure 12. 2D 15N- and 1H- HSQC titration experiment results (1:2.5 ratio)…...……..29

Figure 13. TITAN software results……31

FUTURE STRUCTURAL AND MOLECULAR WORK…….……...33

Figure 14. Amino acid sequence of AKAP18γ(1-75) pET15b ……..35

SIGNIFICANCE- DISRUPTOR PEPTIDE……..36

Figure 15. Diagram illustrating normal Ca^{2+} re-uptake and diagram of future studies with AKAP18γ/I-1 disruptor peptide……..38

CONCLUSION……..39

Figure 16. Illustration of part of AKAP18γ/I-1 binding domain……...41

REFERENCES……..42

LIST OF FIGURES

Figure 1. Cardiovascular disease statistics……..……...….2

Figure 2. Heart failure cycle of events…….……..3

Figure 3. β-adrenergic signaling and AKAP features……..…….6

Figure 4. Schematic illustration of Inhibitor-1 (I-1), with known binding domains and phosphorylation sites…….10

Figure 5. AKAP18 spliced variants……...12

Figure 6. Schematic drawing of AKAP18 role in Ca^{2+} cycling……13

Figure 7. AKAP/I-1 binding is required for I-1 phosphorylation and I-1 binds to the 1-75 segment of AKAP18γ……..……...…18

Figure 8. Illustration of protein purification assays and protein stain of AKAP18γ (1-75) and I-1……..……...…….……21

Figure 9. HSQC spectrum of the 1-75 segment of AKAP18γ and PSIPRED 3.3 protein structure prediction results of AKAP18γ (1-75)……...……...24

Figure 10. PSIPRED 3.3 results for I-1 secondary structure prediction……..…..……25

Figure 11. Two-dimensional HSQC titration experiment……..27

Figure 12. 2D 15N- and 1H- HSQC titration experiment results (1:2.5 ratio)……...……..29

Figure 13. TITAN software results……31

Figure 14. Amino acid sequence of AKAP18γ(1-75) pET15b ……...……..35

Figure 15. Diagram illustrating normal Ca^{2+} re-uptake and diagram of future studies with AKAP18γ/I-1 disruptor peptide……...……..38

Figure 16. Illustration of part of AKAP18γ/I-1 binding domain……...41

LIST OF TABLES

Table 1. Common AKAPs in the heart……8

ABBREVIATIONS

AKAP: A-kinase anchoring protein

PKA: protein kinase A

cAMP: cyclic adenosine monophosphate

LTCC: L-type calcium channel

βAR: β-adrenergic receptor

PP1: protein phosphatase -1

I-1: inhibitor-1

SR: sarcoplasmic reticulum

PLB: phosholamaban

AC: adenylyl cyclase

SERCA2: sarcoendoplasmic reticulum calcium ATPase 2

IDP: intrinsically disordered proteins

IDR: intrinsically disordered regions

DP: disruptor peptides

GST: Glutathione S-transferase

NMR: nuclear magnetic resonance

HSQC: heteronuclear single quantum coherence

DARPP-32: dopamine- and cAMP-regulated phosphoprotein-32

TITAN: TITration ANalysis

HMQC: heteronuclear multiple quantum coherence

AKAP7 (gene name) – AKAP18 (protein name)

ABSTRACT

Cardiovascular disease remains the number one cause of mortality worlwide. Heart failure occurs when the heart does not meet the body's demand. Although significant medical advances have been made, 50% of heart failure patients still die within five years of prognosis, suggesting the need for more research. Alterations of inhibitor-1 (I-1) levels and activity have been associated with heart failure. I-1 PKA-mediated phosphorylation activates its inhibitory activity against PP1, which leads to calcium re-uptake into the sarcoplasmic reticulum (SR). Calcium mishandling plays an important role in cardiac diseases, such as heart failure. Since I-1 is at the top of this signaling pathway, attention has been giving into the function of I-1 in heart failure. AKAP18, a major cardiac scaffold, organizes a multimolecular complex comprised of I-1 and other signaling proteins, which allows the scaffold to regulate Ca^{2+} reuptake into the SR. Our lab was interested in looking into the binding between AKAP18 and I-1.

Our immunoblot analysis with AKAP18 siRNA showed that I-1 was not phosphorylated (by PKA) when AKAP18 was silenced. Previous worked from the lab narrowed the I-1 binding domain to the 1-75 segment of AKAP18 (unpublished work). Our lab looked into this further through protein pulldowns, which showed that I-1-GST immunoprecipitated with AKAP18γ(1-75). Furthermore, I-1 and AKAP18γ(1-75) are highly unstructured, which motivated us to study their binding structurally through NMR spectroscopy. Our 2D ^{1}H-^{15}N-Heteronuclear Single Quantum Coherence (HSQC) titration experiment showed that AKAP18γ(1-75) remained disordered when titrated with I-1. But there were several chemical shifts perturbations in the HSQC spectrum, the most noticeable being a glycine residue we hypothesize to be Gly28. Chemical shift perturbations could be indicative of binding, suggesting the Gly28 might be part of the I-1 binding

domain. Defining the AKAP18/I-1 binding domain can lead to the generation of reagents that disrupt their binding, which could help understand the role of AKAP18/I-1 binding in heart failure, and could open the door to new therapeutic approaches.

CARDIOVASCULAR DISEASE

The heart is a major muscular organ that plays a vital role for human health. As part of the cardiovascular system it pump blood to the rest of the body, supplying peripheral organs and tissues with oxygen and nutrients [8, 9]. Defective heart function has disastrous effects on the human body, and over the years the number of heart disease patients have escalated [1]. According to the American Heart and Stroke Association 2018, cardiovascular disease remains the leading cause of mortality worldwide, claiming more lives than all forms of cancer and Chronic Lower Respiratory Disease combined. In the US, around 2,300 people die of heart disease each day, this averages to 1 death every 38 seconds. Direct and indirect costs of heart disease and stroke is more than $329.7 billion, including both health expenses and lost productivity [1].

Around 9% of the deaths caused by cardiovascular disease can be accredited to heart failure (Figure 1) [1, 9, 10]. Heart failure mortality rates vary between gender (males have higher mortality rates) and racial/ethnic groups (Non-Hispanic Blacks have higher mortality rates) (Figure 1). In the US, approximately 6.5 million people suffer from heart failure, and that number is expected to grow 46% from 2012-2030 [1]. Heart failure occurs when the heart's pumping ability is damaged and cannot meet the body's demand for blood, oxygen and nutrients [1, 9, 10]. This decline in the heart's pumping efficiency and is marked by an interplay between defective cardiomyocyte function and compensatory mechanisms that try to increase the cardiac output, and meet the body's demand [6].

One of these mechanism is done by the sympathetic (adrenergic) nervous system. Initially, to compensate for decreased cardiac output due to, e.g. myocardial infarction, the sympathetic nervous system is activated, stimulating β-adrenergic signaling which leads to an acute

improvement in cardiac output. But long-term β-adrenergic stimulation can lead to adverse effects on the heart, resulting in decompensation and progressive worsening of the heart function (Figure 2) [6]. Even though many new drugs to treat heart failure have been developed over the years, such as β-blockers, coronary revascularization, angiotensin-converting enzyme (ACE) inhibitors, implantable cardioverter- defibrillators, among others [1], the mortality rate of heart failure remains 50% within 5 years [11]. Marking the need for the development of better therapeutic interventions for heart failure.

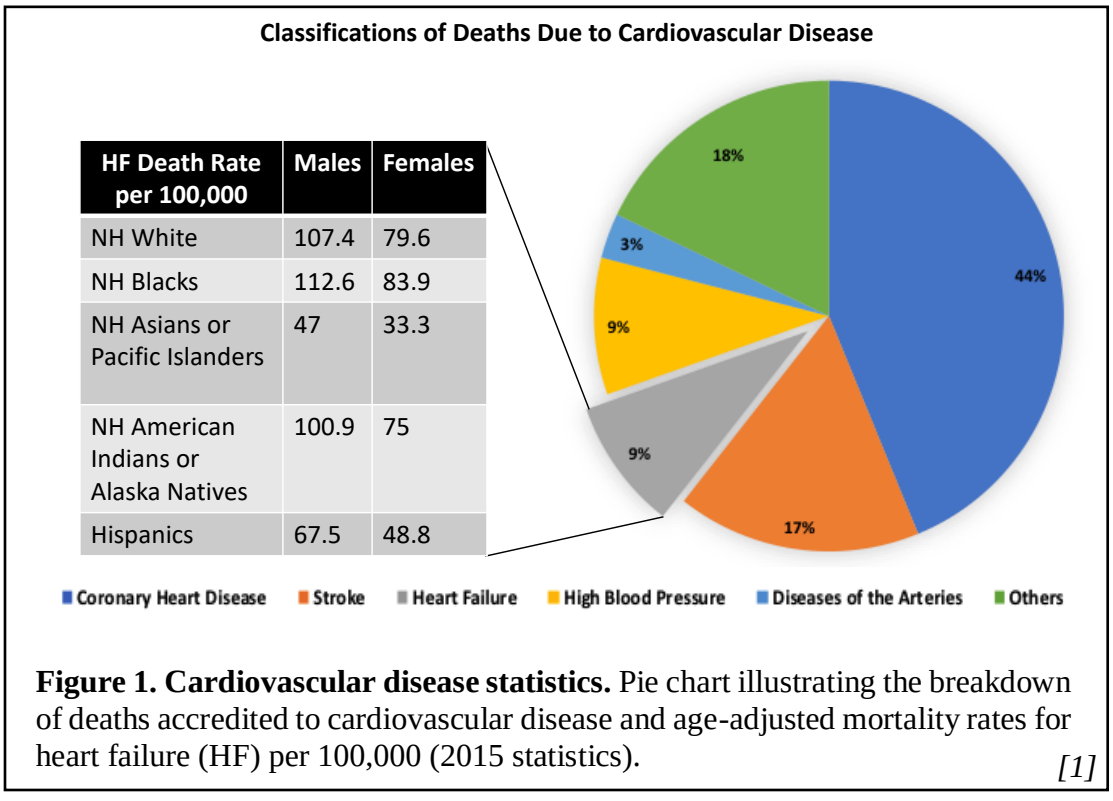


Figure 1. Cardiovascular disease statistics. Pie chart illustrating the breakdown of deaths accredited to cardiovascular disease and age-adjusted mortality rates for heart failure (HF) per 100,000 (2015 statistics). *[1]*

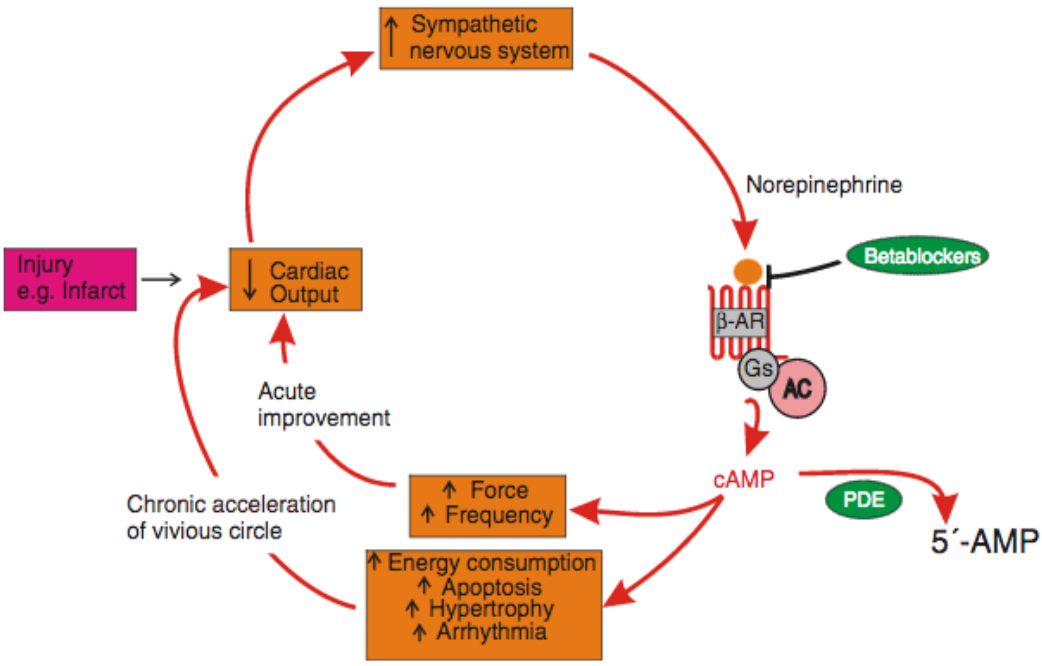


Figure 2. Heart Failure cycle of events. Diagram depicting how the sympathetic nervous system and β-adrenergic receptors work to improve cardiac output. Long-term stimulation of this compensatory mechanism result in progressive worsening of cardiac function. [6]

A-KINASE ANCHORING PROTEINS (AKAPS)

In order to better treat heart failure, it is necessary to study the heart at the cellular level. And one of the key compensatory mechanisms of the heart in response to stress is activation of sympathetic nervous system and β-adrenergic receptors. The cardiomyocyte is equipped with β-adrenergic receptors that can bind to the agonists (e.g. norepinephrine) released as a result of stress [6].This binding results in a confirmational change and the activation of a small G-protein which then activates adenylyl cyclase (AC), an enzyme that generates cAMP. The main target of cAMP is Protein Kinase A or PKA. PKA is a serine/threonine kinase with broad specificity, composed of a regulatory dimer, with each regulatory subunit binding a catalytic subunit. When two molecules of cAMP bind to each regulatory subunit, a confirmational change occurs and the catalytic is released and can phosphorylate substrates (Figure 3A) [12].

Extensive research has shown PKA activity is a critical component to heart disease development [13]. However, because PKA has so many targets, it has become clear that localization of this kinase to the right place in the cell, at the right time, is necessary. This is accomplished via scaffold proteins known as A-Kinase Anchoring Proteins or AKAPs. These scaffolds work by forming protein complexes and coordinating signaling activity, which provides specificity and efficiency [14]. More than 50 AKAPs have been identified including splice variants [12, 15]. Although AKAPs differ structurally [12, 14], they collectively work as scaffolds, bringing proteins in close proximity to their targets, facilitating their signaling transduction.

And there are a few criteria used to characterize proteins as AKAPs. First, a major feature is their conserved amphipathic helix, around 14-18 residues long, that form the PKA binding domain, which gives AKAPs the ability to anchor the regulatory subunits of PKA in close

proximity to its targets (Figure 3B) [15, 16]. Another important feature of AKAPs, is their ability to anchor signaling molecules to specific subcellular localization through a targeting sequence (Figure 3B). Different types of AKAPs have been localized at the endoplasmic reticulum, mitochondria, nuclear membrane, plasma membrane, and dendrites, among others, through fractionation and immunohistochemical analysis [14]. Furthermore, AKAPs anchor enzymes not just to specific organelles, but also facilitate protein activation through specific pools of signaling molecules. Different localized pools of cAMP were first shown in the late 1970s, when two different G-protein coupled receptor agonists increased cAMP in the heart, but only one of them increased glycogen phosphorylase activity and heart contraction [17, 18]. Since PKA is a major target of cAMP, AKAPs anchor cAMP-mediated events to specific subcellular compartments. This not only organizes cAMP signaling processes but also separates signaling pathways within the same compartment, providing spatial and temporal control [14].

AKAPs are also multivalent (Figure 3B), capable of anchoring a variety of signaling molecules including kinases, phosphatases, small GTPases, phosphodiesterases, adenylyl cyclases, transmembrane receptors, transcription factors and ion channels [19, 20]. This feature gives AKAPs the ability to bring signaling complexes to specific compartments inside the cells. Overall, AKAPs allow specificity, localization and spatiotemporal control of signaling events throughout the human body. And over the years, researchers have diverted their attention to investigating how anchored signaling pathways are impaired in disease, bringing AKAPs and its binding partners to the table as potential therapeutic targets.

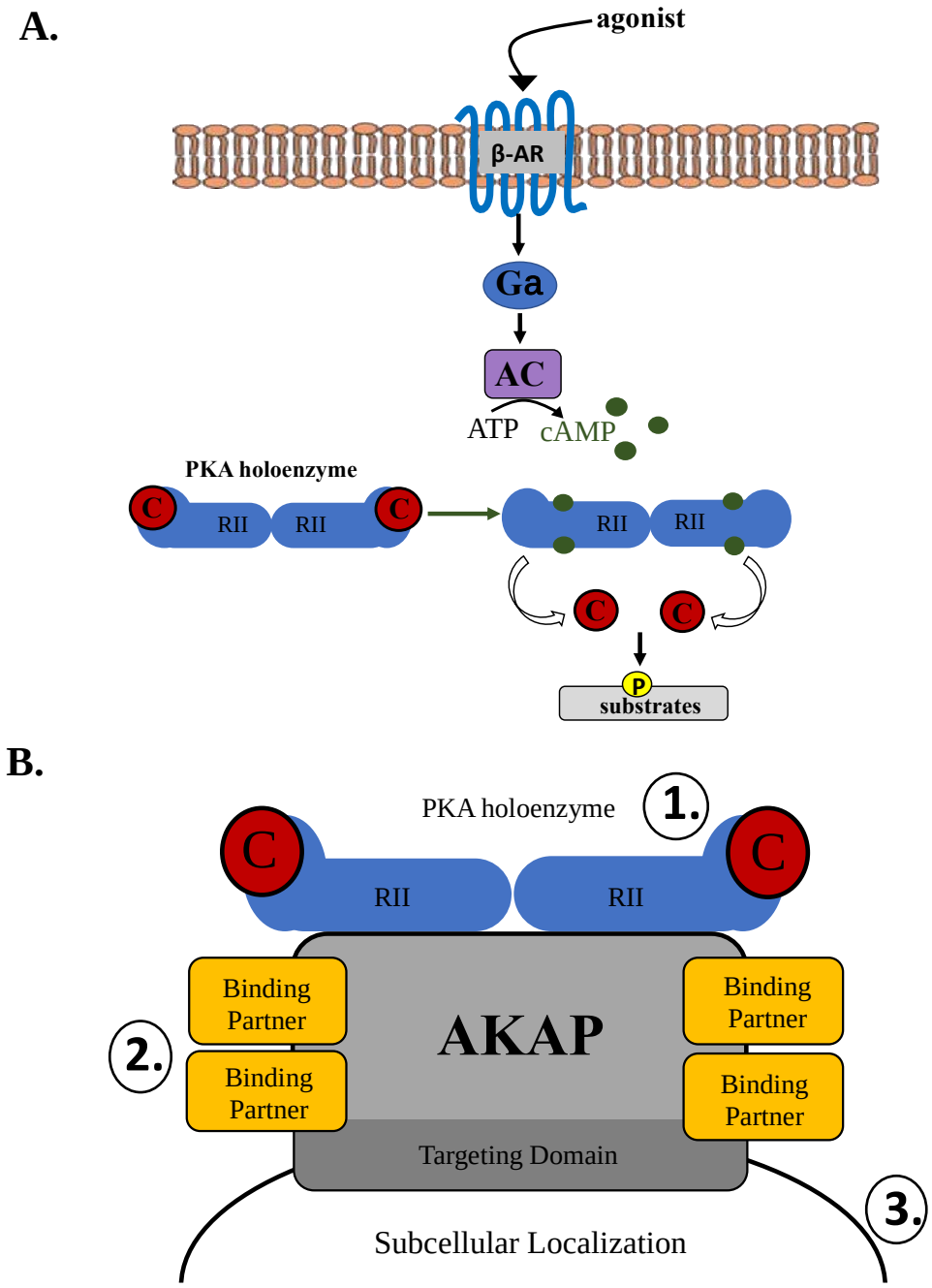


Figure 3. β-adrenergic signaling and AKAP features. A) Diagram illustrating β-adrenergic signaling. B) AKAPs contain 3 crucial features: 1. A conserved amphipathic helix, that allows binding of the regulatory subunits of PKA. 2. AKAPs are capable of anchoring many different signaling molecules. 3. AKAPs carry targeting sequences that allow the anchoring of entire signaling complexes to specific subcellular localizations. B) Diagram illustrating β-adrenergic signaling [6].

AKAPS IN THE HEART

AKAPs ability to form localized multiprotein complexes lead researchers to investigate the role of scaffolds in the induction of heart disease. Around 17 AKAPs have been discovered in the heart [21] (Table 1). Localized at specific subcellular compartments, they regulate many cardiac functions including heart rhythm, action potential duration, calcium movement, cardiac contraction and relaxation, as well as play an essential role in homeostasis, adaptation and pathophysiology [22]. Among the AKAPs present in the heart, AKAP18 remains one of the scaffolds most studied. AKAP18 resides both in the plasma membrane and sarcoplasmic reticulum, facilitating calcium cycling, controlling cardiac contraction and excitation [15].

Yotiao, also known as AKAP 9, coordinates a protein complex around KCNQ1 potassium channel. Regulation is enabled through kinases, phosphodiesterases and phosphatases that are anchored to yotiao, to ensure proper function of the channel. Disrupting the binding between yotiao and KCNQ1 was shown to lead to cardiac arrhythmias [23-25]. AKAP79 is another scaffold that binds and regulates L-type calcium channels [26]. AKAP-lbc was shown to coordinate protein kinase D (PKD1), an important kinase in cardiac remodeling [27, 28]. AKAPs have also been reported in the nucleus (mAKAP and AKAP95), where they facilitate and intrinsically control nuclear signaling [20, 23, 29]. There are many other AKAPs in the heart [23], and their capability to provide spatio-temporal control of entire signaling complexes could give insight into the mechanism of action of cardiovascular diseases, opening the doors for novel therapeutic approaches.

Table 1. Common AKAPs in the heart.

Gene Name	Protein Name	Localization	References
AKAP1	D-AKAP1/2	Outer mitochondrial membrane	[30, 31]
AKAP5	AKAP79	Plasma membrane	[26, 32, 33]
AKAP6	mAKAP	Nuclear envelope	[34, 35]
AKAP7	AKAP15/18	Plasma membrane & sarcoplasmic reticulum	[5, 36]
AKAP8	AKAP95	nucleus	[23, 29]
AKAP9	yotiao	KCNQ1 potassium channel	[23, 24]
AKAP12	gravin	β_2AR	[37]
AKAP13	AKAP-lbc	Actin cytoskeleton	[38, 39]

PROTEIN PHOSPHATASE INHIBITOR-1 (I-1)

AKAPs target PKA to its substrates in order to increase specificity and efficiency in cardiac signaling, motivating reserachers into studying Inhibitor-1 (I-1), a PKA phosphorylation target in cardiomyocytes. I-1 was first reported in the late 1970s and was identified as the first endogenous inhibitor of PP1. I-1 is expressed in all mammalian tissues with the highest expression in pancreas, brain skeletal muscle, heart muscle and kidneys. I-1 is a disordered, soluble protein (~19kDa, 171 amino acids) comprised of many charged amino acids, e.g. proline and glutamate. It is highly stable to heat, low pH organic solvents and detergents [3, 7]. Sequence homology between rat and rabbit tissues showed that its NH_2-terminus region (~ first 50 amino acids), which includes its PKA-mediated phosphorylation site (Thr35), are highly conserved, whereas its COOH-terminus showed more variability [40]. Up to this date, only three I-1 phosphorylation sites have been proven experimentally: Thr35, Ser67 and Thr75. Phosphorylation at Thr35, by PKA, activates I-1 inhibitory activity against PP1, while phosphorylation at Ser67 and/or Thr75 by PKCα suppresses I-1 inhibitory activity. I-1 activation is also regulated by phosphatases, PP2A and PP2B dephosphorylate I-1 at Thr25 (Figure 4) [3, 7].

PP1 is a major cardiac protein phosphatase, and inhibition of PP1(by I-1) leads to Ca^{2+} re-uptake into the sarcoplasmic reticulum (SR), a huge calcium store [2, 3]. Calcium reuptake is required for cardiac relaxation. Interestingly, calcium cycling dysregulation is a hallmark of heart failure [41]. Increased PP1 activity has been linked to heart failure in human and rat models. Furthermore decreased I-1 levels and decreased phosphorylation of I-1 (at Thr35) has been reported in human hearts and animal models as well [3]. So because excessive PP1 activity, possibly caused by lack of I-1 function, may contribute to heart failure and possibly other heart

diseases, means that a more complete understanding of the function of I-1 in the heart is needed, to provide better therapeutic approaches. Additionally, the fact that AKAPs work by forming multiprotein complexes, which includes PKA and its targets raised the following question in the cardiac field: Does I-1 bind to an AKAP in the heart? This question was discussed and investigated by Singh and colleagues a few years back. Through rat heart incubations, pulldown assays and western blots they found that I-1 binds directly to AKAP18γ [4].

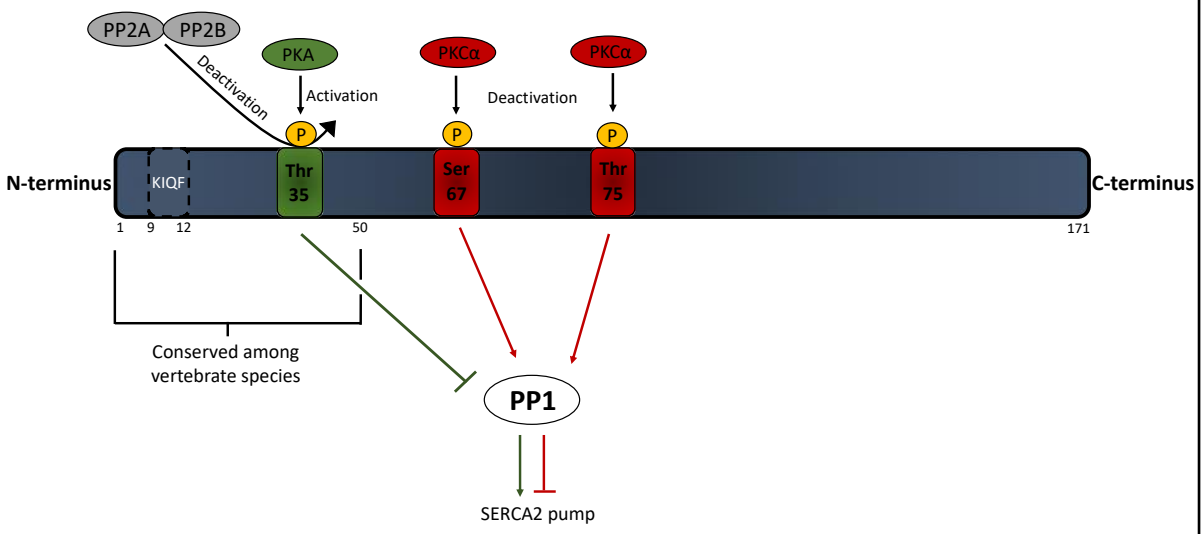


Figure 4. Schematic illustration of Inhibitor-1 (I-1), with known binding domains and phosphorylation sites. The first N-terminus 50 amino acid are conserved among different vertebrate species, while the C-terminus contains more variability. The KIQF motif of I-1 mediates its binding with PP1. The three phosphorylation sites (Thr35, Ser67 and Thr75) regulate its activity. PKA-mediated phosphorylation of Thr35 activate its inhibitory activity towards PP1, promoting Ca^{2+} reuptake into the SR through the SERCA pump. PP2A and PP2B deactivate this signaling by dephosphorylating I-1 at its Thr35 site. Another way of deactivating I-1 activity is through PKCα-mediated phosphorylation of Ser67 and/or Thr75. Deactivation of I-1 inhibitory activity promotes PP1 activation which result in SERCA2 pump inhibition. *[7]*

AKAP18

AKAP18 is highly expressed in the brain and the heart [23]. Encoded by the *AKAP7* gene, it produces 4 known splice variants: alpha, beta, gamma and delta (Figure 5). AKAP18α (also known as AKAP15), is a 18 kDa protein derived from the smallest transcript variant. It is targeted at the plasma membrane of cardiomyocytes and skeletal muscle cells [42] through myristoylation and palmitoylation of its N-terminus, giving AKAP18α the ability to regulate L-type calcium channel (LTCC) after βAR stimulation [15]. Studies in skeletal muscle cells showed that AKAP18α interacts with LTCC through C-terminal domain of the α1 subunit of LTCC [43]. Although these calcium channels are mainly voltage- dependent, channel activity is potentiated by phosphorylation of AKAP18α -bound PKA (Figure 6A) [42, 44]. Disruption of AKAP18α and LTCC binding in mice showed loss of sensitivity to cAMP stimulation, and lead to cardiac hypertrophy [23].

AKAP18β, a 20kDa protein, is structurally and functionally similar to AKAP18α. The additional 24 amino acids facilitate its binding to the apical membrane of polarized epithelial cells, and further investigations are needed to address AKAP18β function at these sites [36]. Not only does AKAP18 coordinate calcium movement at the plasma membrane, but its longer isoforms AKAP18 (δ and γ) are localized at the sarcoplasmic reticulum. Sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2) plays an essential role in calcium cycling by coordinating calcium re-uptake into the sarcoplasmic reticulum. Lygren and colleagues showed that AKAP18δ forms a signalosome containing PKA, phoshpolamban and SERCA2. Phospholamban (PLB) is a negative regulator of the SERCA2 pump. In its dephosphorylated state PLB binds to SERCA2 and inhibits

calcium re-uptake into the sarcoplasmic reticulum. SERCA2 suppression is blocked when PLB is phosphorylated by PKA at Serine 16 (Figure 6B) [5].

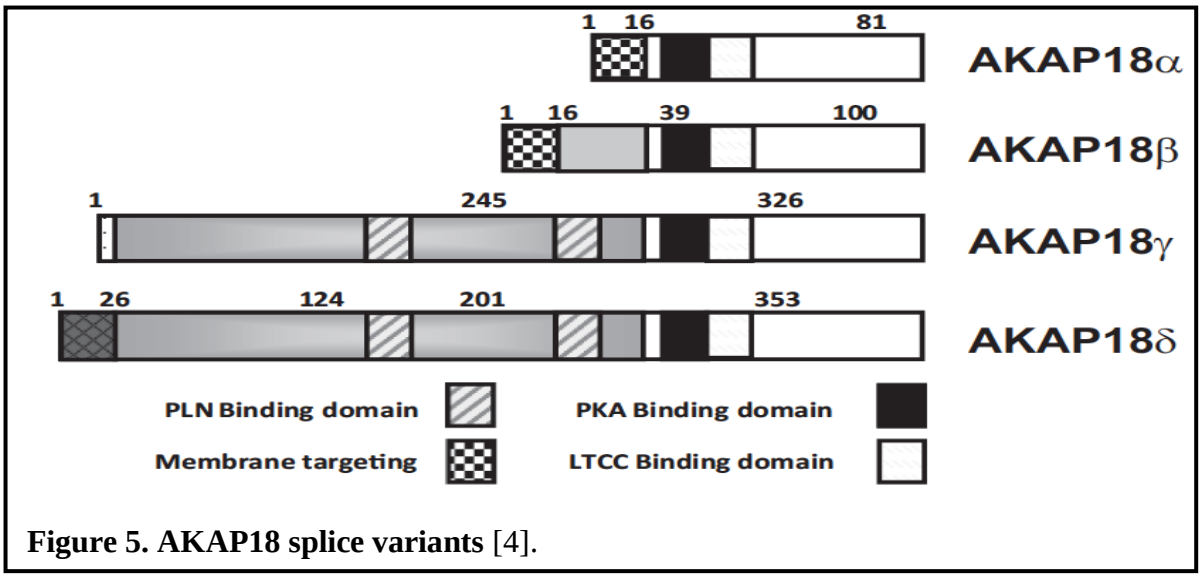


Figure 5. AKAP18 splice variants [4].

Dysregulation of SERCA2 and PLB activity has been connected heart failure [5]. Moreover, there are other molecules associated with the other long isoform, AKAP18γ: protein phosphatase 1 and inhibitor-1, that further control the SERCA2 pump [4]. Singh and colleagues, identified the long isoform AKAP18γ as the scaffold that directly binds to I-1[4]. They were also able to show that AKAP18γ binding to PKA faciliatates I-1 phosphorylation, as well as PP1 inhibition, showing the importance of the scaffold in these signaling events. Displacement of PKA from AKAP18γ attenuated I-1 phosphorylation at Thr 35 site, therefore preventing the effective inhibition of PP1 activity [4] This finding prompted our lab to study the the significance of AKAP18γ and I-1 binding in I-1 phosphorylation. Marking the first question this project wanted to address: Is AKAP18γ/I-1 binding important for I-1 PKA-mediated phosphorylation?

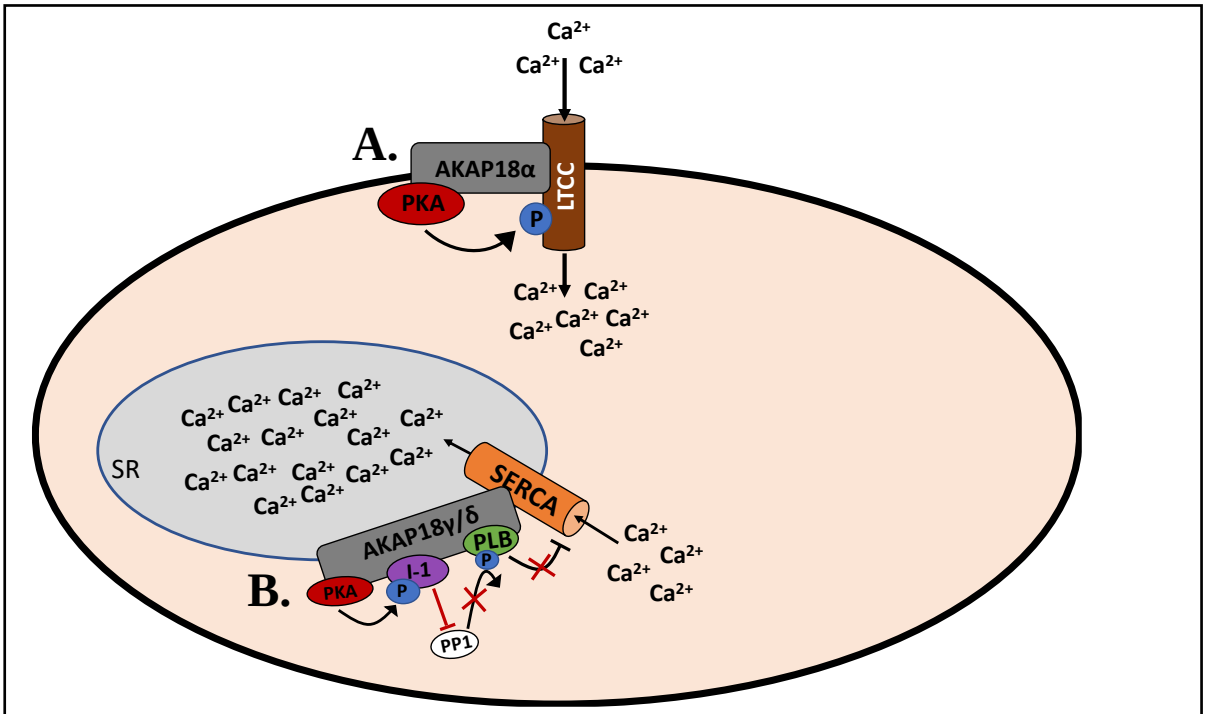


Figure 6. Schematic drawing of AKAP18 role in Ca^{2+} cycling. A) The shorter isoform, AKAP18α, is targeted to the plasma membrane through myristoylation and palmitoylation, where it facilitates L-type Calcium Channel (LTCC) phosphorylation increasing its conductance. B) The longer isoforms AKAP18 (γ and δ) are targeted to the sarcoplasmic reticulum (SR) where they form a protein complex comprising of sarcoendoplasmic reticulum calcium ATPase 2 (SERCA2), phospholamban (PLB), PKA, protein phosphatase 1 (PP1) and inhibitor-1 (I-1). AKAP18 γ/δ orchestrates PKA-mediated I-1 phosphorylation, which activates I-1 inhibitory activity against PP1, maintaining PLB in its phosphorylated form resulting in SERCA2 activation and subsequent movement of Ca^{2+} into the SR.
[2-5]

METHODS

AKAP18 siRNA

For siRNA experiments neonatal rat cardiomyocytes, transfected with AKAP18 siRNA, were stimulated with 100nM isoproterenol for 5 minutes. Immunoblots to determine I-1 phosphorylation were done with a phospho-specific antibody which recognizes Thr35, the I-1 residue phosphorylated by PKA.

Protein Purification

For protein purification assays, LB cultures were started with the gene of interest. The vectors used were I-1-GST, AKAP18γ(1-75) pET15b and I-1 pET15b. Protein expression was induced with 1M IPTG for 5 hours. Cultures were centrifuged for 5 minutes at 6,000 rpm and the pellets were frozen for 1 hour. Frozen pellets were thawed and lysed with 1M PMSF and either binding buffer (20mM HEPES, 0.5mM NaCl, pH 7.4) or GST buffer (recipe below). Binding buffer was used to purify His-tagged proteins and GST buffer was used to purify GST-tagged proteins. The lysate was left rocking at 4°C for 5 hours. Afterwards lysate is centrifuged for 15 minutes at 8,000 rpm, and the collected supernatant is incubated with its respective beads overnight. For His-tagged proteins, Ni beads were used and for GST-tagged proteins GST beads were used. The beads are washed 3X with its respective buffer, ran through SDS-PAGE and stained with Coomassie Blue. Western blots were also performed on the samples (antibody that recognize the His tag of AKAP18γ and an I-1 antibody) to ensure that our proteins of interest were expressed.

Protein Pulldowns

For protein pulldown experiments, the 1-75 segment of AKAP18γ (His-tagged) was bacterially expressed, purified, and Ni beads were stripped off with 1M Imidazole. AKAP18γ (1-75) was

incubated overnight with bacterially expressed and purified I-1-GST (GST beads were used to pulldown the 1-75 segment of AKAP18γ). Binding between the AKAP18γ(1-75) and I-1 was determined through western blot with a His antibody that recognized the His tag on the AKAP18γ(1-75) protein. GST beads (with a GST tag) were used as controls and protein pulldown experiments were done in triplicate. Input were also used as controls, to ensure AKAP18γ(1-75) expression in control experiments.

NMR Spectroscopy

To prepare protein samples for NMR, ^{15}N-labeled AKAP18γ(1-75) pET15b and unlabeled I-1 pET15b proteins were bascterially expressed, purified and stripped off their Ni beads with 1M Imidazole. They were both dialyzed to a NMR appropriate binding buffer (20mM, 100mM NaCl, pH 7.0) and concentrated (^{15}N-labeled AKAP18γ(1-75) - 0.3mM and unlabeled I-1 - 1.52mM) using Amicon® Ultra-4 Centrifugal Filter Units**.** Before concentrating, 1M DTT was added to I-1 pET15b and AKAP18γ (1-75) pET15b samples at 1:1,000 dilution, to avoid AKAP18γ(1-75) dimerization. To study the interaction of ^{15}N-labeled AKAP18γ(1-75) and unlabeled I-1, a two-dimensional ^{1}H-^{15}N-HSQC titration experiment was performed on a 500 MHz NMR spectrometer. ^{15}N-labeled AKAP18γ(1-75) (0.3mM) was titrated with unlabeled I-1 (1.52mM) at a 1:0.6, 1:1 and 1:2.5 ratio. All NMR spectra were processed and analyzed using NMRbox software and the glycine chemical shift perturbation was further analyzed through the TITAN software. All NMR experiments were performed at 25°C .

PSIPRED 3.3

The primary amino acid sequence of the 1-75 segment of AKAP18γ and I-1 was inserted into the PSIPRED 3.3 software to predict their respective secondary structure.

GST Buffer Recipe

50 mls 100% glycerol

5 mls Triton X-100

25 mls 1M Tris pH 8.0

15 mls 5M NaCl

5 mls EDTA

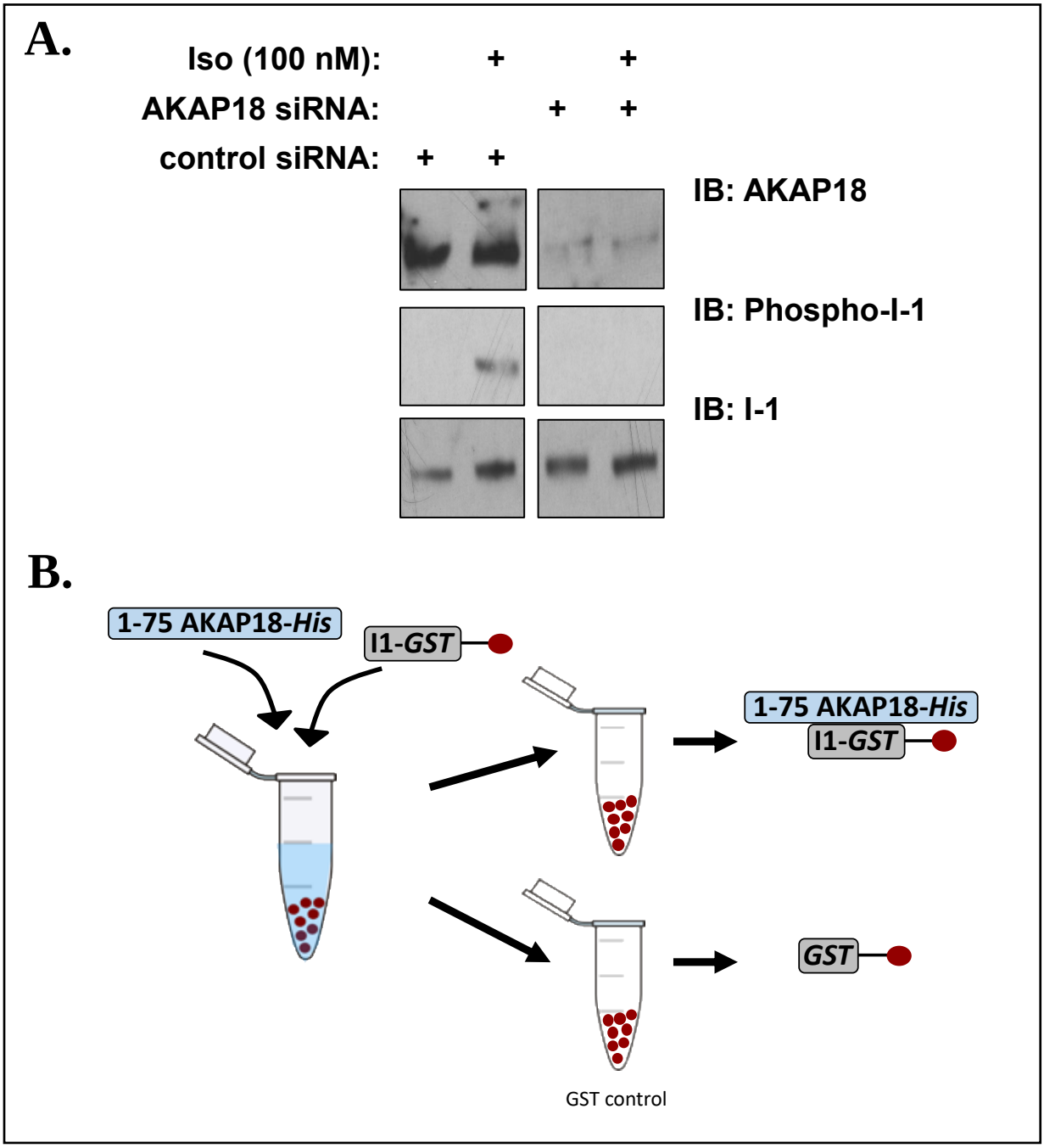
400 mls H_20

RESULTS

Previous work demonstrated that PKA displacement from AKAP18γ attenuated I-1 phosphorylation at Thr 35 site, therefore preventing the effective inhibition of PP1 activity [4]. The importance of AKAP18γ and PKA binding prompted our lab to study the significance of AKAP18 and I-1 binding in I-1 phosphorylation. Neonatal rat cardiomyocytes were transfected with an AKAP18 siRNA, stimulated with isoproterenol (100nM) for 5 minutes and I-1 phosphorylation was determined by western blot with a phospho-specific antibody that recognizes Thr35 site (I-1 residue phosphorylated by PKA). Immunoblot results showed that isoproterenol stimulation did not phosphorylate I-1 in the cells that were transfected with AKAP18 siRNA, compared to control siRNA where I-1 phosphorylation, through isoproterenol stimulation, was observed (Figure 7A). These results suggests that AKAP18 and I-1 binding might be required for I-1 phosphorylation, which is supported by literature depicting AKAP18 as a scaffold orchestrating signaling activity between its binding partners [4, 5].

The possibility that AKAP18 and I-1 might be crucial for I-1 phosphorylation motivated our lab to look into the binding domain of AKAP18 and I-1. Previous mapping work had narrowed down AKAP18/I-1 binding domain to the 1-75 segment of AKAP18 (unpublished work), this was further studied through protein pulldowns. The 1-75 segment of AKAP18γ (His-tagged) was bacterially expressed and purified, Ni beads were stripped off with Imidazole. AKAP18γ(1-75) was incubated with bacterially expressed and purified I-1, which was tagged with GST (Figure7B). The binding between the 1-75 segment of AKAP18γ and I-1 was determined through western blot with a His antibody that recognized the His tag on the 1-75 segment of AKAP18γ. Results showed that AKAP18γ(1-75) precipitated only with I-1-GST, and not to the GST tag alone, further

demonstrating the specificity of the binding between the 1-75 segment of AKAP18γ and Inhibitor-1 (Figure 7C).



C.

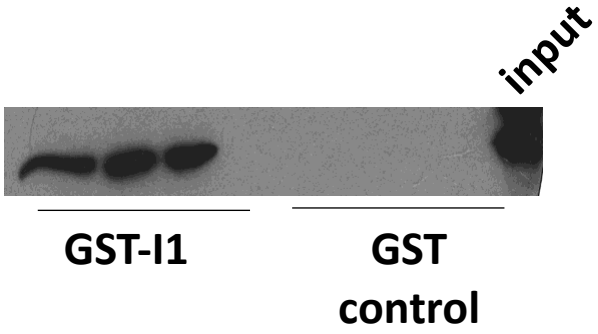


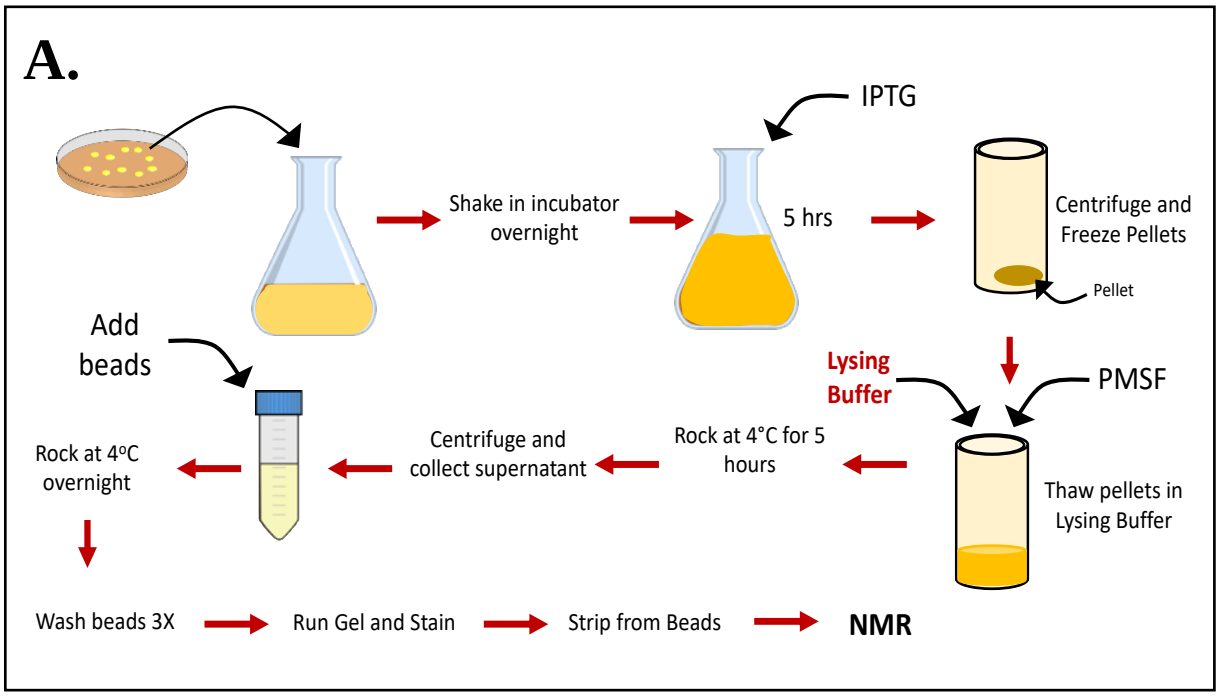
Figure 7. AKAP18/I-1 binding is required for I-1 phosphorylation and I-1 binds to the 1-75 segment of AKAP18γ. A) Rat neonatal cardiomyocytes transfected with AKAP18 siRNA and stimulated with isoproterenol (100nM) showed no I-1 phosphorylation, compared to control siRNA. **B)** Diagram illustrating the purified protein pulldown experiment. **C)** Western blot showing that purified AKAP18γ(1-75) His-tagged only precipitated with GST-tagged I-1, and not with the GST tag alone. His antibody was used to determine binding.

AKAP18/INHIBITOR-1 BINDING DOMAIN

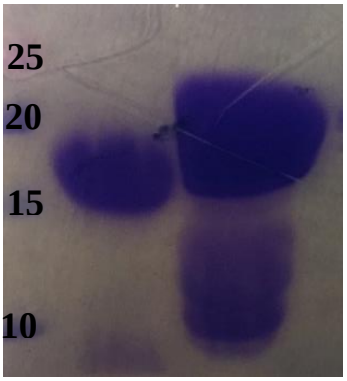
Our lab wanted to look even further, we were interesting in knowing what region I-1 binds to the 1-75 segment segment of AKAP18γ. This marks the second question this project wanted to address: defining the binding domain between I-1 and the 1-75 segment of AKAP18γ. Although I-1's tight regulation has been the subject of many studies, it's I-1 protein structure that gained attention over the years. I-1 is a disordered, comprised of many charged amino acids [7]. An interesting fact about AKAP18 is that it N-terminus region is predicted to be disordered as well [45]. Unstructured proteins can also be referred as intrinsically disordered proteins (IDPs) or intrinsically disordered regions (IDRs) [46, 47]. In the past research was guided by the common concept that a protein's function is closely related to its 3D structure, which still remains true. But over the years, it has been found that certain proteins don't fit this common criteria [46].

These proteins called Intrinsically Disordered Proteins (IDPs), they function even though they lack tertiary contacts and usually do not have stable secondary structure. They lack bulky hydrophobic residues (e.g. valine, leucine, tryptophan, among others) which usually form the hydrophobic core of folded proteins. These IDPs are dynamical and flexible, which permits them to adopt distinct conformations upon binding to different partners [46, 47]. A paper paper published a few years ago looked at the binding between PP1 and another inhibitor called Spinophilin. Spinoliphin is a neuronal regulatory protein that targets PP1. Since PP1 regulates neuronal synapse, Spinophilin plays an important role in learning and memory. And the Spinoliphin PP1-binding domain is highly unstructured. After performing NMR in these proteins they found out that Spinophilin folds when binding to PP1[48].

There is a direct interaction between I-1 (IDP) and the 1-75 segment of AKAP18γ (IDR). And the fact that these disordered proteins may adopt different conformation (even fold) upon binding to other proteins, suggest that the 1-75 segment of AKAP18 might do the same upon binding to I-1. And that's the third question our lab wanted to address. Does the 1-75 segment of AKAP18 fold upon binding to I-1? So these are the last two questions this project wanted to elucidate. First, our lab was interested in pinpointing the I-1/AKAP18 binding domain. Second, since the 1-75 segment of AKAP18 is disordered, we are curious to find out if AKAP18(1-75) folds upon binding to I-1, another disordered protein, as suggested by literature [46, 47]. Both questions were addressed through protein purifications (Figure 8A and B) and NMR spectroscopy.



B.



AKAP18γ (1-75) pET15b I-1 pET15b

Figure 8. Illustration of protein purification assays and protein stain of AKAP18γ (1-75) and I-1. A) Bacterially expressed proteins are purified and stripped from its beads according to the illustration above (for more information see Methods). **B)** Protein stain of AKAP118γ(1-75) and I-1, stripped from its beads.

NMR SPECTROSCOPY RESULTS

First, we wanted to observe the unbound structure of the 1-75 segment of AKAP18γ, so ^{15}N-labeled AKAP18γ (1-75) was bacterially expressed, purified and stripped off its beads (Figure 8A and B), to then analyze by NMR. The two dimensional ^{1}H- and ^{15}N- Heteronuclear Single Quantum Coherence (HSQC) spectrum showed that the 1-75 segment of AKAP18γ was indeed unstructured, which is characterized by crowded and narrow peaks in the ^{1}H dimension (Figure 9A). The structure of the 1-75 segment of AKAP18γ was also looked at through PSIPRED 3.3 protein structure prediction server, which can predict secondary structures from its amino acid sequence alone. PSIPRED accuracy is measured by Q3 scores, which calculates the percentage of residues predicted correctly and the PSIPRED 3.2 achieved a Q3 score of 81.6% [49].

Its results showed that there is a high confidence of prediction for a helix between residues of 49 through 56, and a beta sheet between amino acids 67 and 68 of AKAP18γ(1-75). All other helixes predicted have a low confidence of prediction, suggesting that this segment of AKAP18γ is unstructured (Figure 9B), which supports our HSQC results on unbound AKAP18γ (1-75). We also looked into the structure of I-1 through the PSIPRED server (Figure 10), and the results showed only two helixes (residues 22-32 and 81-92) with high confidence of prediction and the majority of the rest of the I-1 protein was predicted to contain random coils (with high confidence of prediction), which indicate lack of a stable secondary structure consistent with literature depicting I-1 as a highly unstructured protein [3, 7].

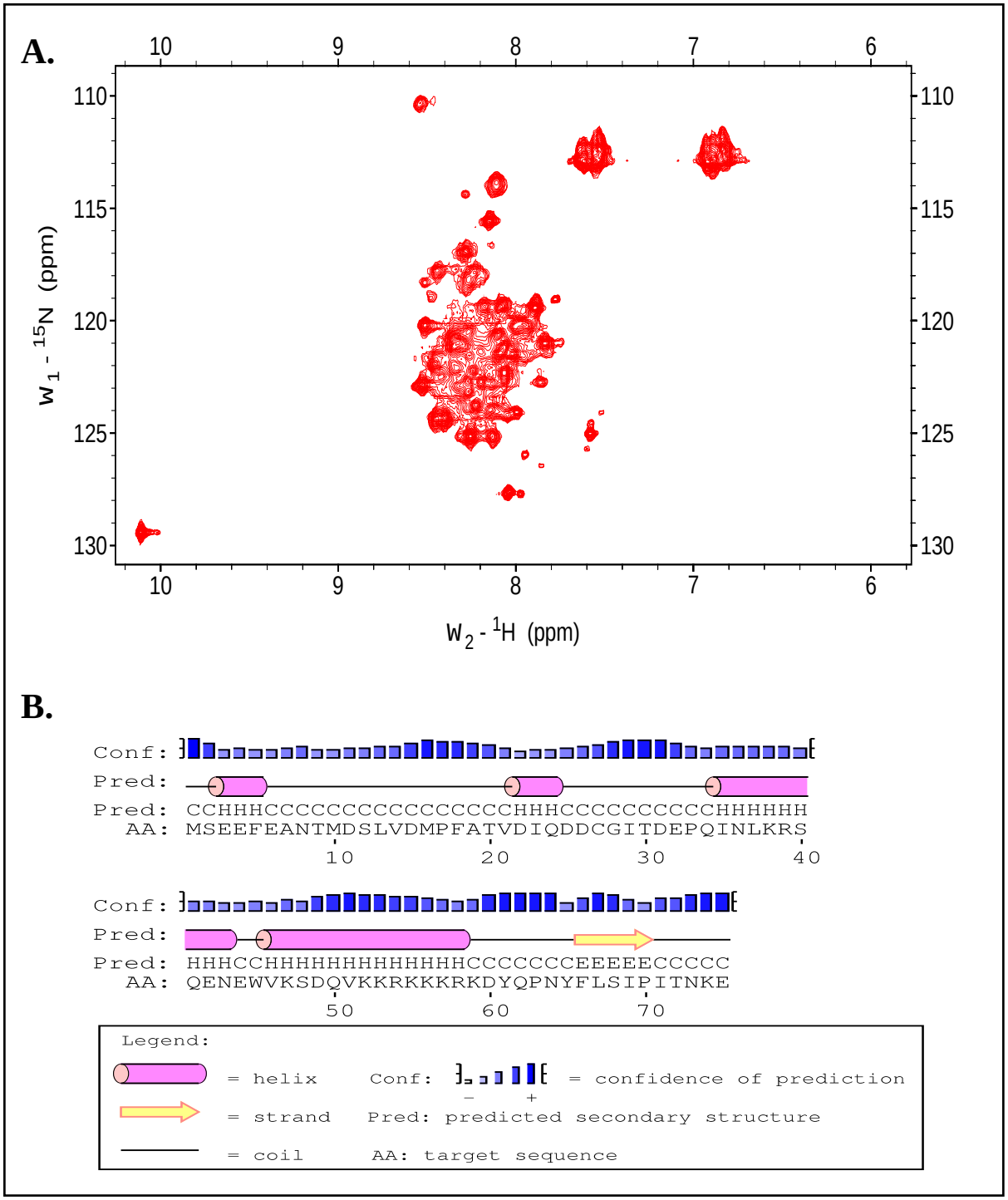

A.
10
9
8
7
6
110
115
120
125
130
w_1 - ^{15}N (ppm)
w_2 - ^{1}H (ppm)
B.
Conf:
Pred:
Pred: CCHHHCCCCCCCCCCCCCCCCHHHCCCCCCCCCCCHHHHHH
AA: MSEEFEANTMDSLVDMPFATVDIQDDCGITDEPQINLKRS
10
20
30
40
Conf:
Pred:
Pred: HHHCCHHHHHHHHHHHHHCCCCCCCEEEEECCCCC
AA: QENEWVKSDQVKKRKKKRKDYQPNYFLSIPITNKE
50
60
70
Legend:
= helix
= strand
= coil
Conf:
– +
= confidence of prediction
Pred: predicted secondary structure
AA: target sequence

Figure 9. HSQC spectrum of the 1-75 segment of AKAP18γ and PSIPRED 3.3 protein structure prediction results of AKAP18γ (1-75). A) Agglomerated and narrow peaks in the ^{1}H dimension characterize the 1-75 segment of AKAP1γ as an intrinsically disordered region. **B)** PSIPRED results show high confidence of prediction for a helix (approximately residues 49-56) and a beta sheet (residues 67-68), the rest seems unstructured.

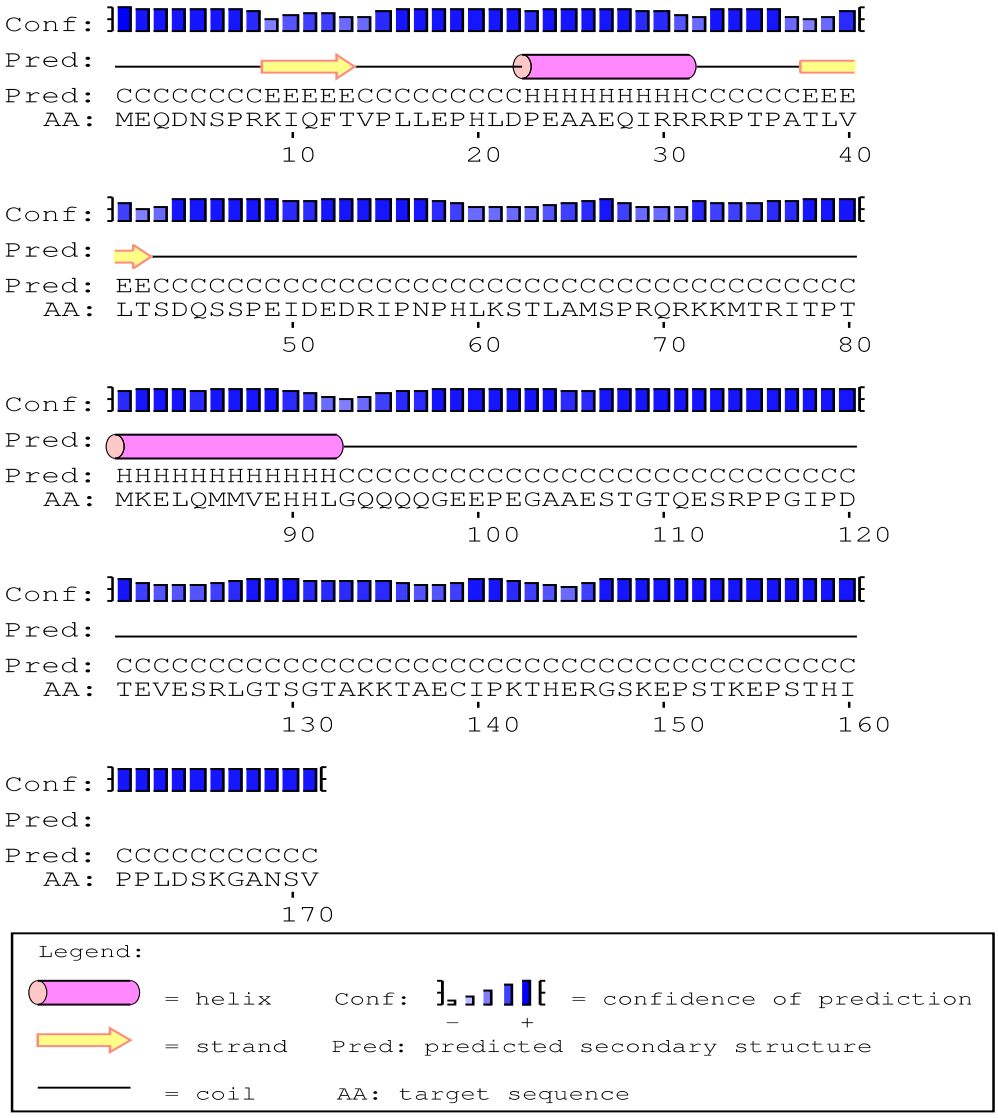


Figure 10. PSIPRED 3.3 results for I-1 protein structure prediction. PSIPRED 3.3 protein secondary structure server showed high confidence of prediction for two helixes and random coils (which occupy the majority of the protein structure).

It was also interesting to observe that one of the helixes predicted for I-1 was located between residues 22-32. A previous study detected a transient helix between residues 22-29 of dopamine- and cAMP-regulated phosphoprotein-32 (DARPP-32), another PP1 inhibitor with high levels of amino acid sequence identity in the first 50 residues with I-1 [50, 51], demonstrating the high efficiency of prediction of the PSIPRED 3.3 protein structure prediction server. After looking into unbound AKAP18γ (1-75), a two-dimensional ^{15}N- and ^{1}H- HSQC titration experiment (1:0.6, 1:1 and 1:2.5) was performed between ^{15}N-labeled AKAP18γ(1-75) and unlabeled I-1. Despite research suggesting that intrinsically disordered proteins may fold upon interaction with binding partners, our results showed that the structure of AKAP18γ (1-75) remained disordered when titrated with I-1 at a 1:0.6, 1:1 and 1:2.5 ratio (Figure 11).

Although our lab did not perform amino acid assignments, some residues reside in specific locations in the HSQC spectrum e.g. tryptophan, glycine, asparagine and glutamine. Tryptophan residues are located on the bottom left of the HSQC spectrum between ppm 10 and 11 at the ^{1}H dimension. Our lab observed only one peak which is consistent with the only tryptophan residue in the 1-75 segment of AKAP18γ, Try45, where no changes were observed in the Try45 peak upon I-1 titration (Figure 12A). Asparagine and glutamine side chain usually generate two peaks in the same ^{15}N dimension (between 110 and 115 ppm) but in different ppm values in the ^{1}H dimension. No changes were observed in neither of these peaks when titrated with I-1 at a 1:0.6, 1:1 and 1:2.5 ratio (Figure 11 and 12B). To answer one of our questions: does AKAP18(1-75) fold upon binding to I-1? No, but there were several chemical shifts perturbations observed. And this is where our lab starts addressing the other question: Defining the I-1 binding domain of AKAP18.

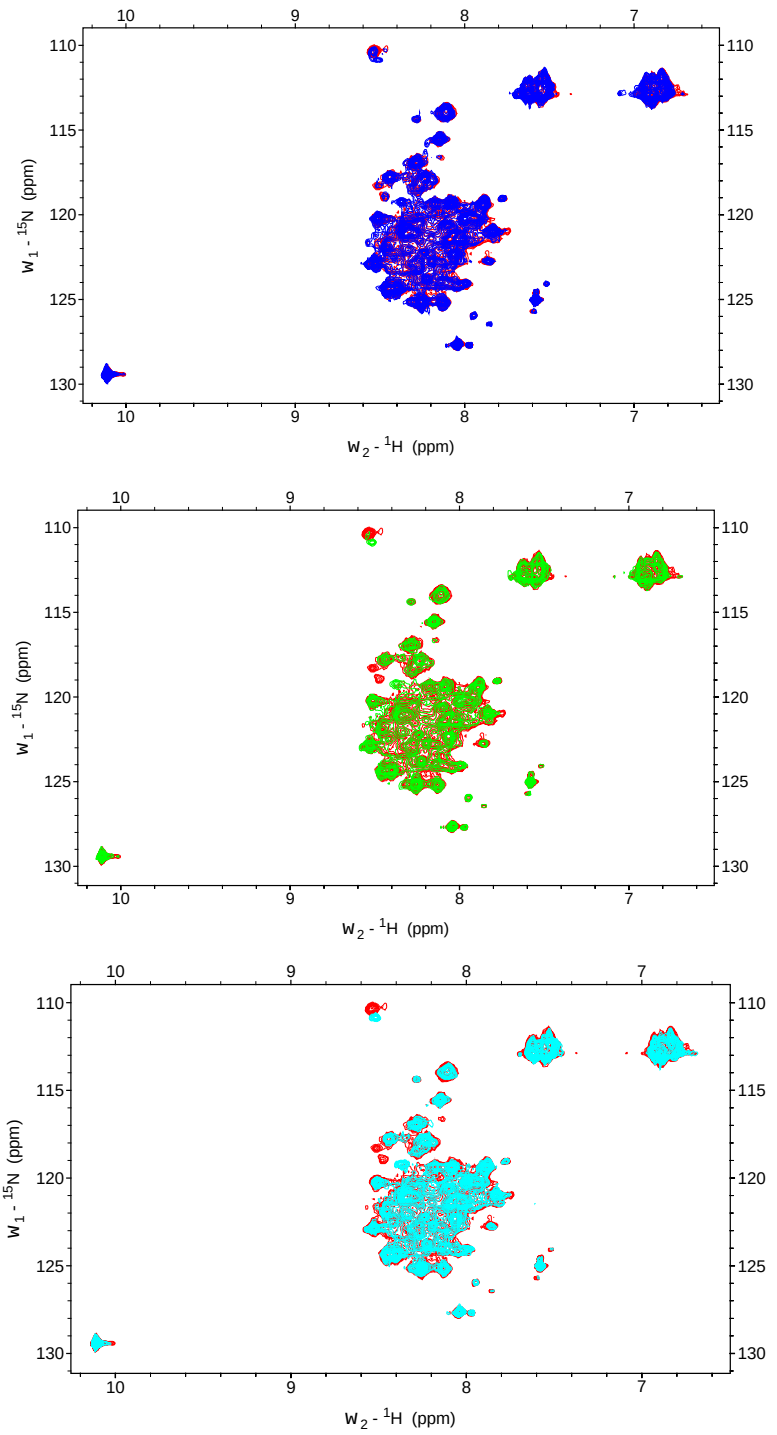


Figure 11. Two-dimensional HSQC titration experiment. The overall structure of ^{15}N-labeled AKAP18γ (1-75) remained disordered when titrated with I-1 at a 1:0.6 (top panel, blue), 1:1 (middle panel, green) and at a 1:2.5 (bottom panel, turquoise) ratio. Unbound AKAP18γ (1-75) is represented in red.

One of them was located between ppm values 118-120 (^{15}N dimension) and between ppm values 8.2-8.6 (^{1}H dimension). We clearly observed that two strong peaks of unbound ^{15}N-labeled AKAP18γ(1-75) disappear when titrated with I-1, and one strong peak appears upon titration with I-1, most noticeable at 1:2.5 ratio (Figure 12C). The specifics of the observed peak changes need to be studied further, to determine which amino acid residues undergo these chemical shift perturbations. The other chemical shift perturbation was observed in the region were glycine residues are located. One strong peak is observed in unbound ^{15}N-labeled AKAP18γ(1-75) (shown in red) and upon titration with I-1 (shown in turquoise) AKAP18γ(1-75) experienced a chemical shift change in what our lab hypothesizes to be glycine 28, the only glycine residue in the 1-75 segment of AKAP18γ (Figure 12D). Future work is needed to determine if this peak belongs to Gly28 in the AKAP18γ and not the other three glycine residues in the tag and linker of AKAP18γ(1-75), used for the bacterial purification assays.

The location of this glycine residue (isolated from the agglomerated peaks in the middle of the HSQC spectrum) permitted us to analyze this residue (possibly Gly28) through a software tool called TITAN (TITration ANalysis) [52]. Through the HSQC spectrum and TITAN data we could determine the rate of exchange of our titration experiment. During a slow exchange, a gradual change in peak position occurs as one titrates the ligand in. In a fast exchange, the free peak signal progressively disappears, and the bound peak signals appears in another position in the HSQC spectrum [52, 53]. Our titration data suggest that in the Gly28 peak, and the changes in chemical shifts observed in the middle of the HSQC spectrum, a slow exchange occurred as the free peak signal (AKAP18γ (1-75)) gradually disappeared when I-1 was titrated in, another peak signal appeared (Figure 11 and 13A).

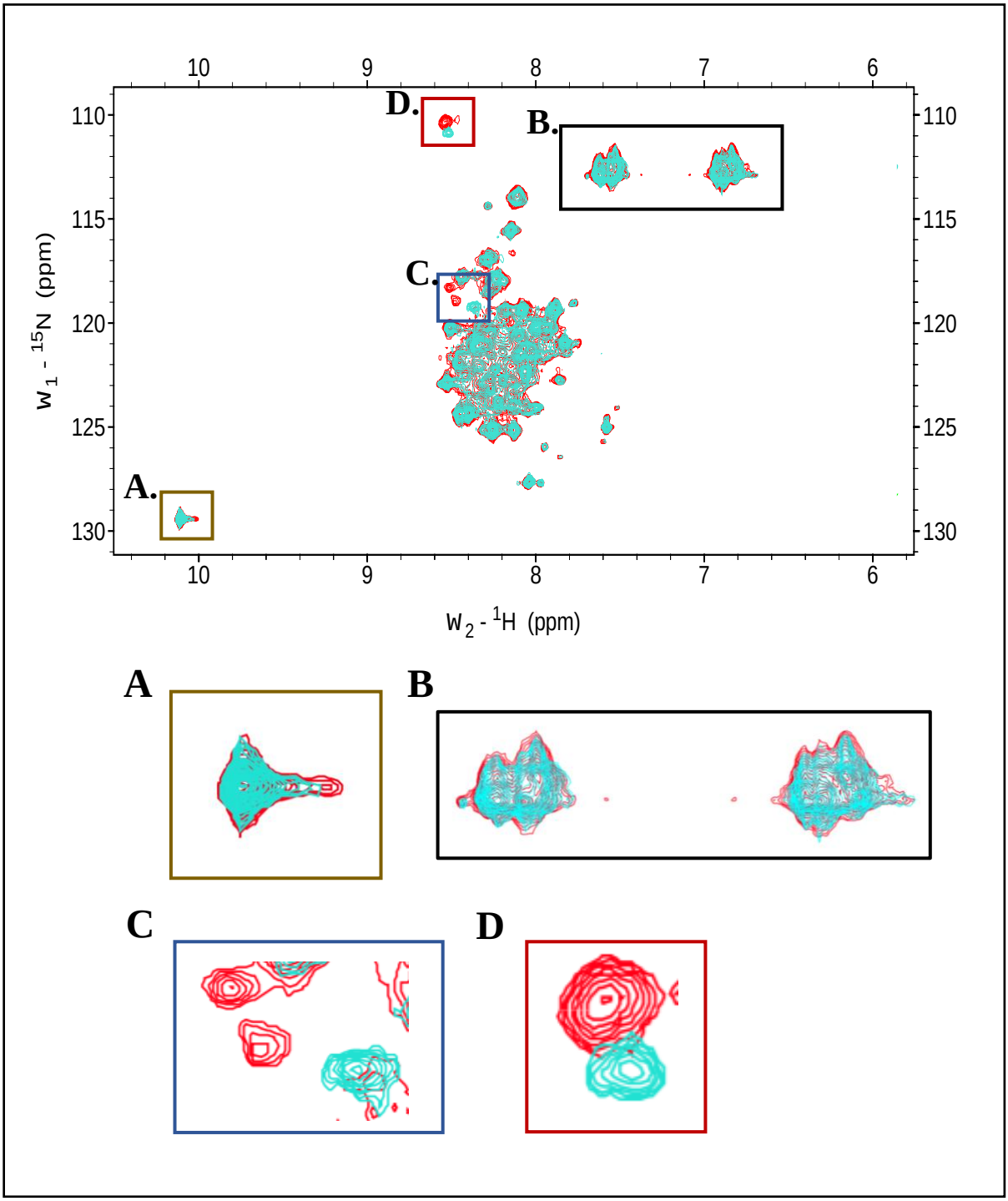

10
9
8
7
6
110
115
120
125
130
D.
B.
C.
A.
w_1 - ^{15}N (ppm)
w_2 - ^{1}H (ppm)
A
B
C
D

Figure 12. Two-dimensional ^{15}N- and ^{1}H- HSQC titration experiment results (1:2.5 ratio). A) AKAP18γ(1-75) only tryptophan residue, Trp 45, did not undergo any changes upon titration with I-1. **B)** No changes were observed in asparagine and glutamine side chain peaks. **C)** Two strong peaks of unbound AKAP18γ(1-75) (red) disappeared and one strong visible peak appeared upon titrating with unlabeled I-1. More research is needed to identify the amino acids involved in these chemical shift perturbations. **D)** In the section of the HSQC spectrum where glycine residues reside, another chemical shift perturbation was observed, which we hypothesize to be Gly28, and could be indicative of binding. Red = unbound ^{H}N-labeled AKAP18γ, turquoise = AKAP18γ(1-75) titrated with I-1 at a 1:2.5 ratio.

This change in chemical shift in the Gly28 peak was better studied through the TITAN software, where we could observe the two different peak positions between free and bound AKAP18γ (1-75). A change in chemical shift was immediately observed when AKAP18γ(1-75) was titrated at a 1:0.6 ratio, and the strongest bound peak signal was seen when AKAP18γ(1-75) was titrated at a 1:2.5 ratio (Figure 11 and Figure 13A). The TITAN software is a novel software used to study titration experiments. It can simulate HSQC and HMQC pulse programs and researchers are able to fit their experimental data with a range of binding models. If the selected model fits with experimental data it could give insight into the binding mechanism, as well as indicate the dissociation constant (K_d) (11). The binding model that fit our titration data was a simple two-state model and the dissociation constant (K_d) given was 34 +/- 5 μM (Figure 13A).

The 3D viewer was used to observe and ensure that our titration data fit the binding model selected. Our experimental titration data is represented in gray and the model data is represented in red (Figure 13B). Chemical shifts perturbation could be indicative of binding. Although further work is needed to identify the rest of the residues interacting directly with AKAP18γ (e.g. amino acid assignments, mutagenesis studies, etc.), our study provides the first step towards mapping the I-1 binding site of AKAP18γ and our lab proposes that Gly28 forms part of this binding domain.

Overall, our data suggests that AKAP18γ/I-1 binding is crucial for I-1 phosphorylation (and I-1 activation), and defining their binding domain could give insight into the importance of AKAP18γ/I-1 binding in calcium cycling and heart disease.

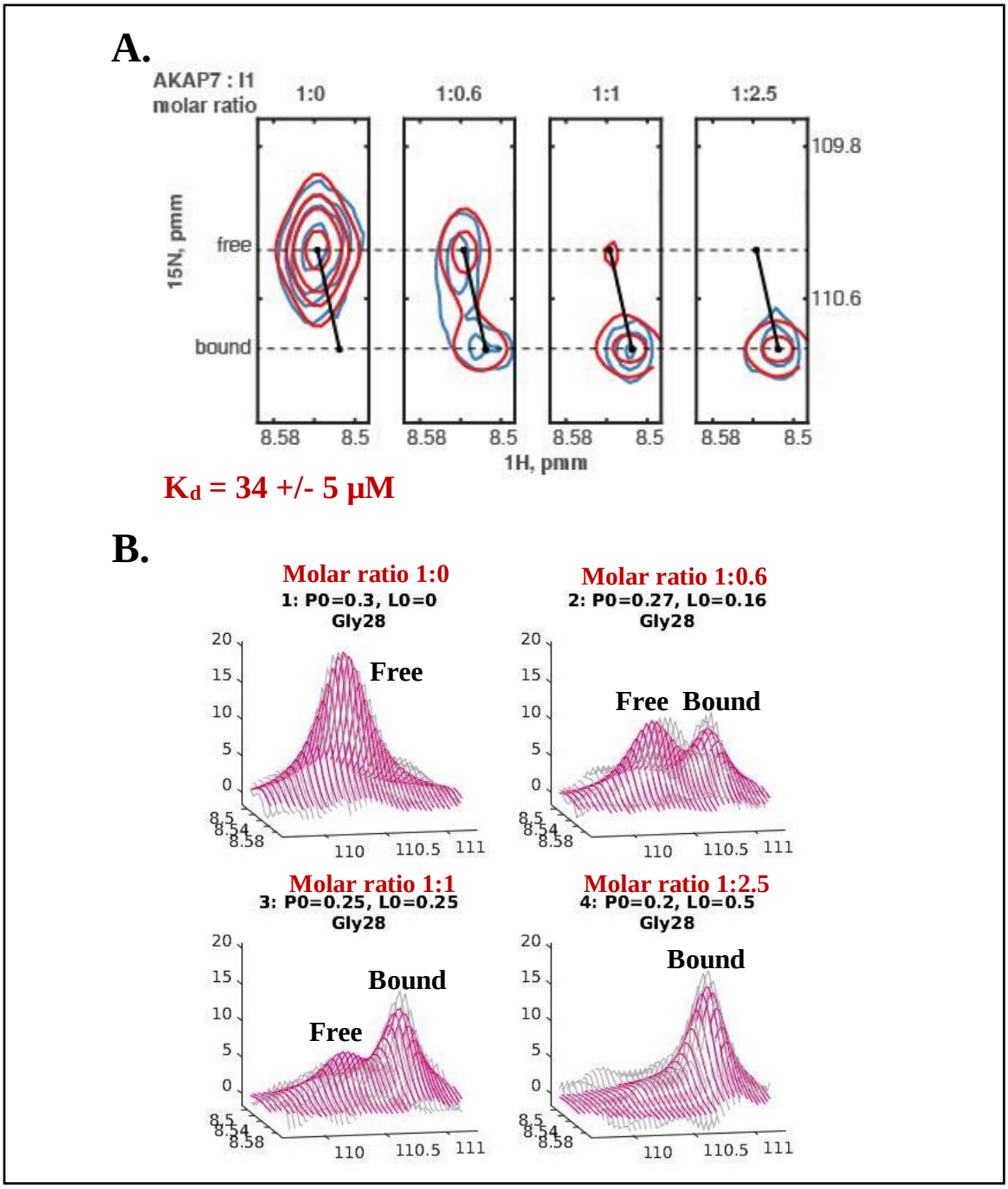


Figure 13. TITAN software results. A) Detailed visualization of Gly28, in the HSQC spectrum, as it undergoes a change in chemical shift when titrated with I-1 at a 1:0.6, 1:1 and 1:2.5 ratio. The dissociation constant (K_d)provided from the TITAN software was 34 +/- 5 µM. **B)** 3D viewer that indicated that our titration data (gray) fit the binding model (red) provided through the TITAN software. P0= AKAP18γ(1-75) concentration, L0= I-1 concentration.

FUTURE STRUCTURAL AND MOLECULAR WORK

To our knowledge these are the first structural reports of AKAP18γ and I-1 binding. Although the 1-75 segment of AKAP18γ remained disordered, the chemical shift perturbation observed in a glycine peak on ^{15}N labeled 1-75 AKAP18γ surely sparked our interest. Chemical shift perturbations in the HSQC spectrum could be indicative of binding. And our lab hypothesizes that the chemical shift observed in the HSQC spectrum belongs to Gly28, since it is the only glycine residue found in the 1-75 AKAP18γ amino acid sequence, but there are three other glycine residues found in its His tag and linker, which is why further work needs to be done (Figure 14). Mutagenesis studies could be a great way to elucidate which glycine residue is causing this peak change, e.g. mutating Gly28, with an alanine residue. After titrating the mutated (G28A)^{15}N-labeled 1-75 AKAP18γ with unlabeled I-1, if no change occurs in the allotted space where Gly28 resides, then it would support our hypothesis that the observed peak change was indeed Gly28, and not the glycine residues in the tag and linker.

The other chemical shift perturbations observed in our titration experiments could not be analyzed through the TITAN software due to the agglomerated, narrow peak distribution in the ^{1}H dimension, which is characteristic of unstructured proteins. Furthermore, these chemical shift changes did not occur in residues that have distinct localizations in the HSQC spectrum, which would make them easy to identify, e.g. glycine or tryptophan. Therefore amino acid assignments should be performed in the 1-75 segment of AKAP18γ to properly identify all the amino acid that acquire chemical shift perturbation when titrated with I-1. It would be interest to also perform titrations of ^{15}N-labeled I-1 with unlabeled 1-75 AKAP18γ to address any potential structural changes on I-1 that may arise from I-1 and 1-75 AKAP18γ binding.

I-1 is an intrinsically disordered protein (IDPs), and their dynamic properties suggest the possibility of I-1 folding and/or assuming a different confirmation upon binding to the 1-75 segment of AKAP18γ [46, 47]. Many disordered proteins also undergo post-translational modifications, which "can tune their conformational states and their interaction with their binding partners" [46]. The structure of I-1 has been studied before and its conformation does not experience any apparent change when phosphorylated at Thr35 [7]. Phosphorylation of Thr35 does however increase its binding affinity towards native PP1 [54]. It would be interesting to see if I-1 phosphorylation at Thr35 has any effect on its affinity towards AKAP18γ.

Singh and colleagues demonstrated that although I-1 binds to an AKAP118γ mutant (1-268), which lacks the PKA binding site, I-1 phosphorylation (at Thr35 site) becomes significantly attenuated in isoproterenol-stimulated HEK293 cells compared to I-1 phosphorylation when bound to full-length AKAP18γ [4]. So the lack of I-1 phosphorylation (by PKA) observed in our immunoblots when AKAP18 was silenced by siRNA, not only could be due to the absence of AKAP18γ-I-1 binding, but could also be partly caused by the absence of AKAP18γ-PKA binding. Furthermore PKA activity is highly dependent on AKAP18γ binding, the AKAP18γ mutant (1-268) lacked kinase activity, which explains the decreased phosphorylation at Thr35 site observed in I-1 [4]. But I-1 is not the only PKA target in the multiprotein complex organized by AKAP18γ in the SR. Phospholamban is another PKA substrate whose phosphorylation at Serine 16 releases PLB's inhibition towards SERCA2, thus activating the Ca^{2+} pump [5]. PKA also has substrates outside the AKAP18γ/PLB/I-1/PKA complex at the SR. For example, Ryanodine Receptors (RyRs) are major calcium release channels in the SR that are targets for phosphorylation by PKA

[55]. PKA is also known to phosphorylate LTCCs, troponin I and myosin binding protein C, which are all essential proteins for cardiac contraction [2].

Since there are multiple PKA substrates involved in cardiac function, silencing AKAP18 might attenuate PKA activity, affecting the modulation of its targets. This brings out the necessity of defining the binding domain between AKAP18γ and Inhibitor-1. Thanks to previous work, our lab narrowed down the I-1 binding domain to the 1-75 segment of AKAP18 (unpublished work). Defining AKAP18γ I-1-binding domain could also clarify whether the chemical shift observed in Gly28 (presumably) in our study is due to Gly28 direct binding to I-1 or if it's a conformational change induced by I-1 binding to residues nearby. Knowledge of its exact binding domain could help investigate the structural implications/mechanism of AKAP18γ-I-1 binding. Does AKAP18γ orchestrate I-1 phosphorylation (at Thr35) by placing I-1 in close proximity to PKA? Or does binding to the scaffold induce a conformation change in I-1, exposing the site for PKA-mediated phosphorylation? Addressing these questions could give insight into the mechanism of I-1 phosphorylation and activation at a structural level.

M**G**SSHHHHHHSS**G**LVPR**G**SHM M S E E F E A N T M D S L V D M P F A T V D I Q D D C G I T D E P Q I N L K R S Q E N E W V K S D Q V K K R K K K R K D Y Q P N Y F L S I P I T N K E

Figure 14. Amino acid sequence of the 1-75 segment of AKAP18γ in pET15b. The 1-75AKAP segment is highlighted cyan and the His tag and linker are represented with the white box. Glycine residues are marked in bold and underlined, glycine 28 is marked in red.

SIGNIFICANCE- DISRUPTOR PEPTIDE

After elucidating the exact binding domain between AKAP18γ and I-1, it would be of great interest to generate an AKAP18γ/I-1 disruptors peptide to study in more depth the importance of this binding. Disrupting binding partners from AKAPs has been studied in the past especially with AKAP-PKA disruptor peptides (10). But as mentioned before PKA has a vast number of substrates, and disrupting PKA from AKAP18 might affect the phosphorylation of its many targets [4]. And a major disadvantage of these peptide disruptors is that they do not interfere with a specific complex, instead they disrupt all AKAP-PKA complexes [2]. Which is why attention has been given to disrupting other AKAP18 binding partners, and why our lab focused on I-1. AKAP18-I-1 disruptor peptides would only affect I-1 at the SR, which is an important element to have in mind since I-1 has been reported to have targets at other cellular compartments outside the SR [7].

I-1 has been associated with eIF2α, a protein involved with protein synthesis, that has been localized to the nucleus. Phospholemman and Na/K-ATPase, which are located at the plasma membrane, are regulated by PP1 and I-1 in cardiomyocytes. I-1 expression has also been shown in renal distal convoluted tubule, which is not a surprise since I-1 is also highly expressed in the kidneys, and a study suggests it may regulate NaCl cotransporter (NCC) activity, a protein that controls arterial blood pressure [7, 56-59]. Targeting the AKAP18-I-1 binding domain would only interfere with the function of I-1 at the SR, without affecting its possible role at other compartments.

AKAP18γ-I-1 disruptor peptides could be a great tool to study I-1 phosphorylation and its inhibitory activity without affecting AKAP18γ expression nor its ability to form protein complexes

at the plasma membrane and at the SR level which facilitate calcium handling. Peptide disruptors could also be used to perform PP1 phosphatase activity assays to correlate lack of I-1 phosphorylation (due to I-1 being displaced from AKAP18γ) with decreased PP1 phosphatase activity, as one would expect since I-1 PKA-mediated phosphorylation activates its inhibitory activity towards PP1 [7]. On a molecular level disrupting the binding between AKAP18γ and I-1 could be used to observe the effects on I-1 phosphorylation and activity, PP1 phosphatase activity, PLB phosphorylation state and SERCA2 pump activity (Figure 15A and B).

Additionally generating AKAP18γ-I-1 disruptor peptides could be of great use to study the importance of this binding in calcium cycling. I-1 is known as a distal β-adrenergic signaling amplifier of PKA activity, being that phosphorylation of I-1 (by PKA) results in activation of SERCA2 pump, followed by calcium re-entry into the SR. Defective calcium handling is observed in cardiac arrhythmias, contractile dysfunction, heart failure, among other cardiac diseases [2]. Our results suggesting that AKAP18γ-I-1 binding is required for I-1 phosphorylation, might point to AKAP18γ-I-1 disruptor peptides as a possible new model for defective calcium handling seen in cardiac diseases (Figure 15A and B). Furthermore, I-1 levels and phosphorylation appeared decreased in human failing hearts and heart failure mouse models. AKAP18γ-I-1 disruptor peptides could help study in more depth the signaling mechanisms involved in heart failure, with the intention of identifying targets that could lead to novel therapeutic approaches for heart failure, as well as other cardiac diseases associated with impaired calcium cycling.

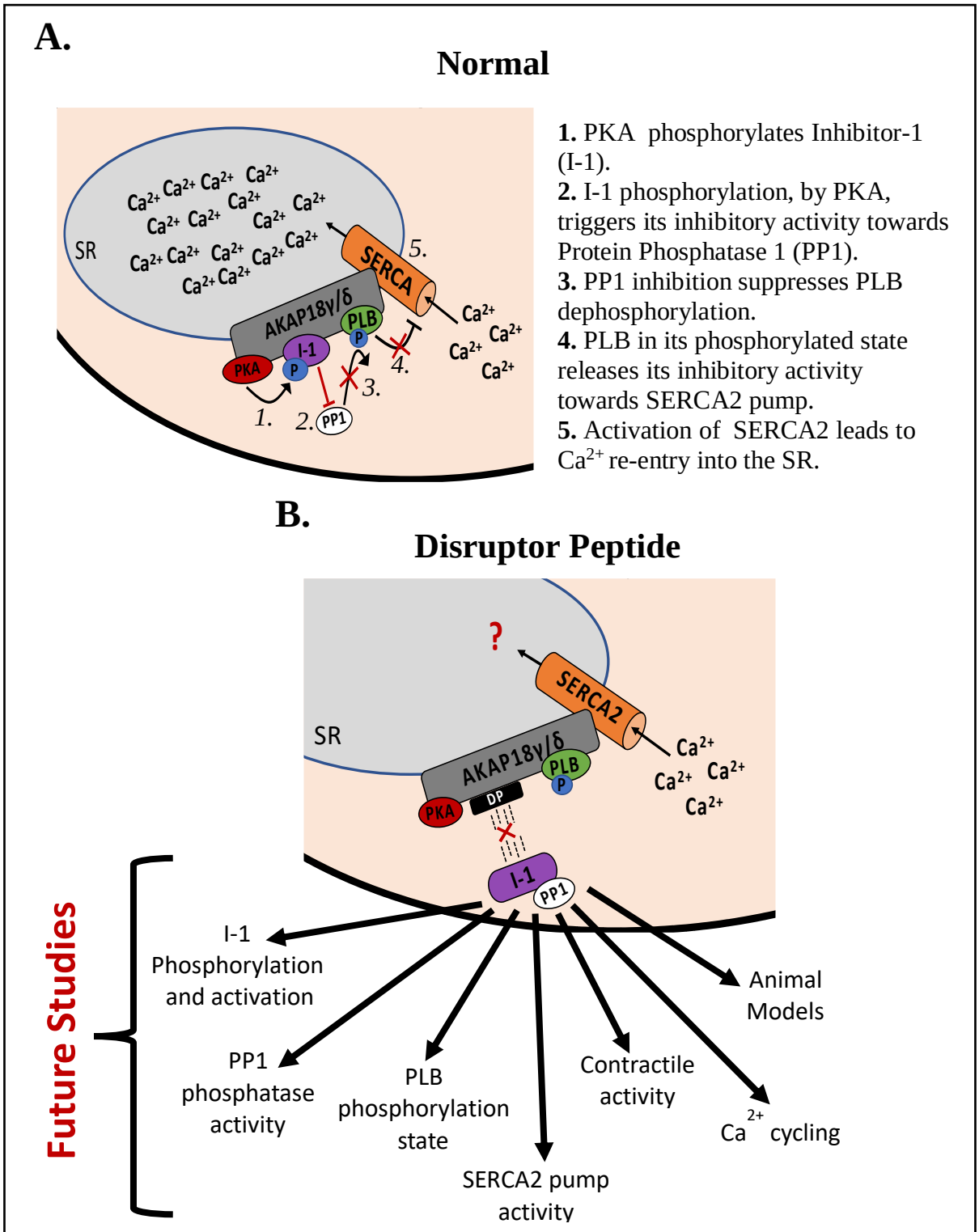


Figure 15. A) Diagram illustrating how I-1 phosphorylation leads to calcium re-entry into the SR under normal conditions. **B)** Diagram illustrating future possible studies with AKAP18γ-I-1 disruptor peptides

CONCLUSION

The heart is a major muscular organ that plays a vital role for human health. Defective heart function has disastrous effects on the human body, and over the years the number of heart disease patients have escalated, marking the need for new targets for therapeutic interventions. AKAP18 plays a critical role in calcium handling in the heart, at the plasma membrane and at the SR. Calcium handling is critical for cardiomyocyte contraction and relaxation, and perturbation in calcium cycling can result in heart disease [2]. Disturbed PLB and SERCA2 activity has been associated with heart failure [5, 60]. The other binding partners of AKAP18 (at the SR) have also been investigated, one of them being I-1, the upstream regulator of SERCA2 pump activity. Altered I-1 levels and phosphorylation (at Thr35) have also been linked with heart failure, making it a potential target for therapeutic approaches [3, 7].

I-1 has not been studied solely at a molecular level but its biochemical properties make I-1 an interesting protein to study at a structural level as well, because I-1 is highly unstructured [3, 7]. Moreover, not only I-1 is an unstructured protein, but AKAP18 contains a disordered region (N-terminus)[45] and previous mapping studies narrowed down AKAP18/I-1 binding domain to the 1-75 segment of AKAP18 (unpublished work). Which brings out this interesting scenario where an unstructured protein and an unstructured region interact, and literature suggest they might undergo conformational changes (even folding) upon binding [46, 47].

Our lab started by first studying the importance of AKAP18/I-1 binding at a molecular level. AKAP18 siRNA experiments showed that I-1 did not get phosphorylated in the cells where AKAP18 was silenced and purified protein pulldowns demonstrated direct binding between I-1 and the 1-75 segment of AKAP18, suggesting that I-1 binding to AKAP18 may be crucial for I-1

phosphorylation and subsequent activation of its inhibitory function. These results motivated us even more to study their interaction at a structural level through NMR spectroscopy. NMR results showed that the 1-75 segment of AKAP18 (labeled with ^{15}N) is in fact unstructured. And contrary to what some people believed, the 1-75 segment of AKAP18 does not fold upon binding to I-1, but it does experience some structural changes. Our most significant finding was a chemical shift observed in the glycine region in the HSQC spectrum. Its isolated and clear signal permitted us to analyze the chemical shift using the TITAN software.

Our lab hypothesizes that the glycine residue the underwent a chemical shift is glycine 28, since it's the only glycine residue in the 1-75 segment of AKAP18. Although further mutagenesis studies will be needed in order to confirm that since there are three more glycine residues in the His-tag and linker. Overall, these results mark the beginning of determining the I-1 binding domain of AKAP18. The chemical shift perturbations observed in the HSQC spectrum of AKAP18γ (1-75) when titrated with I-1 can be indicative of binding, so there is a high probability that Gly28 is part of the AKAP18/I-1 binding domain (Figure 16). Understanding the AKAP18-I-1 binding domain at a structural level could lead to the development of disruptor peptides/reagents, which could open the door to novel therapeutic approaches to combat calcium mishandling seen in cardiac disease such as heart failure.

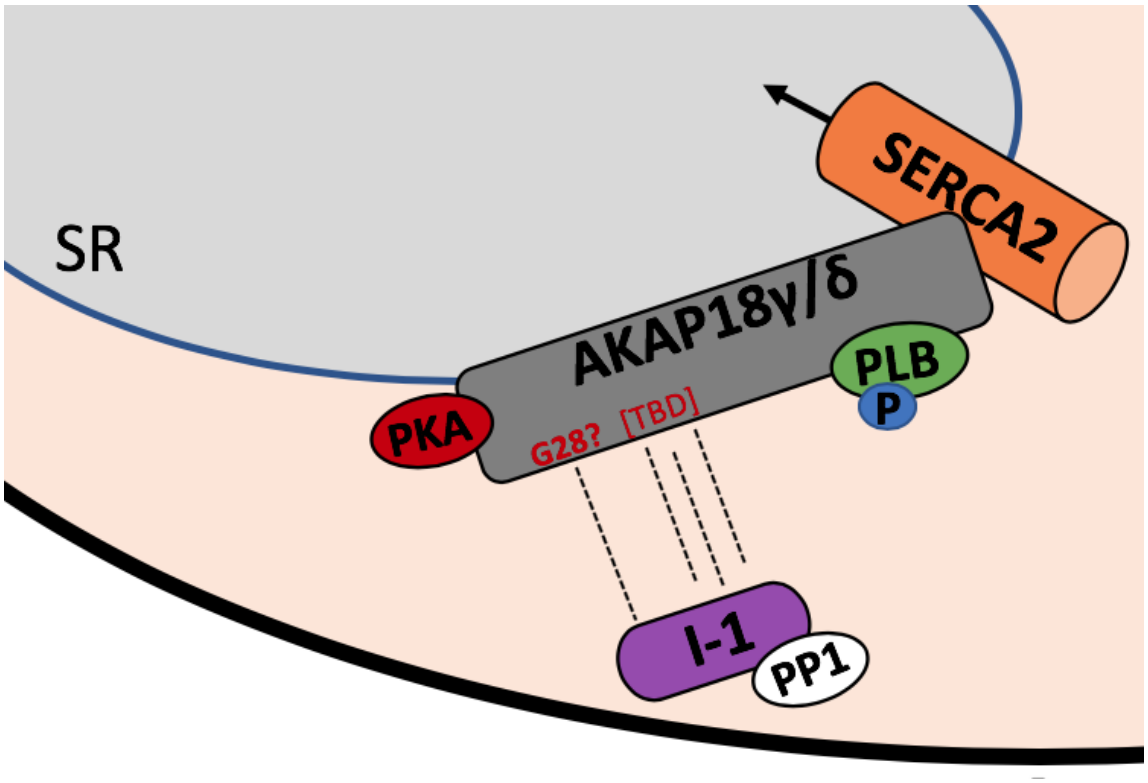


Figure 16. Illustration of part of AKAP18γ/I-1 binding domain. Our results suggest that glycine 28 is part of AKAP18/I-1 binding domain, more studies are needed to determine the rest of the residues involved in AKAP18γ and I-1 binding. TBD = To Be Determined.

REFERENCES

1. Benjamin Emelia, J., et al., *Heart Disease and Stroke Statistics—2018 Update: A Report From the American Heart Association.* Circulation, 2018. **137**(12): p. e67-e492.
2. Lygren, B. and K. Tasken, *The potential use of AKAP18delta as a drug target in heart failure patients.* Expert Opin Biol Ther, 2008. **8**(8): p. 1099-108.
3. Nicolaou, P., R.J. Hajjar, and E.G. Kranias, *Role of protein phosphatase-1 inhibitor-1 in cardiac physiology and pathophysiology.* J Mol Cell Cardiol, 2009. **47**(3): p. 365-71.
4. Singh, A., et al., *The large isoforms of A-kinase anchoring protein 18 mediate the phosphorylation of inhibitor-1 by protein kinase A and the inhibition of protein phosphatase 1 activity.* Mol Pharmacol, 2011. **79**(3): p. 533-450.
5. Lygren, B., et al., *AKAP complex regulates Ca2+ re-uptake into heart sarcoplasmic reticulum.* EMBO Rep, 2007. **8**(11): p. 1061-7.
6. El-Armouche, A. and T. Eschenhagen, *β-Adrenergic stimulation and myocardial function in the failing heart.* Heart Failure Reviews, 2008. **14**(4): p. 225.
7. Weber, S., S. Meyer-Roxlau, and A. El-Armouche, *Role of protein phosphatase inhibitor-1 in cardiac beta adrenergic pathway.* Journal of Molecular and Cellular Cardiology, 2016. **101**: p. 116-126.
8. Suryavanshi, S.V., S.M. Jadhav, and B.K. McConnell, *Polymorphisms/Mutations in A-Kinase Anchoring Proteins (AKAPs): Role in the Cardiovascular System.* Journal of cardiovascular development and disease, 2018. **5**(1): p. 7.
9. Diviani, D., et al., *Emerging roles of A-kinase anchoring proteins in cardiovascular pathophysiology.* Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 2016. **1863**(7, Part B): p. 1926-1936.
10. Yancy Clyde, W., et al., *2013 ACCF/AHA Guideline for the Management of Heart Failure.* Circulation, 2013. **128**(16): p. e240-e327.
11. Mozaffarian, D., et al., *Heart Disease and Stroke Statistics—2016 Update.* Circulation, 2016. **133**(4): p. e38-e360.
12. Jarnaess, E., et al., *Dual specificity A-kinase anchoring proteins (AKAPs) contain an additional binding region that enhances targeting of protein kinase A type I.* J Biol Chem, 2008. **283**(48): p. 33708-18.
13. Gold, M.G., T. Gonen, and J.D. Scott, *Local cAMP signaling in disease at a glance.* Journal of cell science, 2013. **126**(Pt 20): p. 4537-4543.
14. Colledge, M. and J.D. Scott, *AKAPs: from structure to function.* Trends Cell Biol, 1999. **9**(6): p. 216-21.
15. Scott, J.D., C.W. Dessauer, and K. Taskén, *Creating Order from Chaos: Cellular Regulation by Kinase Anchoring.* Annual Review of Pharmacology and Toxicology, 2013. **53**(1): p. 187-210.
16. Esseltine, J.L. and J.D. Scott, *AKAP signaling complexes: pointing towards the next generation of therapeutic targets?* Trends in Pharmacological Sciences, 2013. **34**(12): p. 648-655.
17. Keely, S.L., *Activation of cAMP-dependent protein kinase without a corresponding increase in phosphorylase activity.* Research communications in chemical pathology and pharmacology, 1977. **18**(2): p. 283-290.

18. Hayes, J.S., et al., *Hormonally specific expression of cardiac protein kinase activity.* Proceedings of the National Academy of Sciences of the United States of America, 1979. **76**(4): p. 1570-1574.
19. Welch, E.J., B.W. Jones, and J.D. Scott, *Networking with AKAPs: context-dependent regulation of anchored enzymes.* Mol Interv, 2010. **10**(2): p. 86-97.
20. Passariello, C.L., et al., *mAKAP-a master scaffold for cardiac remodeling.* J Cardiovasc Pharmacol, 2015. **65**(3): p. 218-25.
21. Diviani, D., H. Osman, and E. Reggi, *A-Kinase Anchoring Protein-Lbc: A Molecular Scaffold Involved in Cardiac Protection.* Journal of cardiovascular development and disease, 2018. **5**(1): p. 12.
22. Ercu, M. and E. Klussmann, *Roles of A-Kinase Anchoring Proteins and Phosphodiesterases in the Cardiovascular System.* Journal of cardiovascular development and disease, 2018. **5**(1): p. 14.
23. Redden, J.M. and K.L. Dodge-Kafka, *AKAP phosphatase complexes in the heart.* J Cardiovasc Pharmacol, 2011. **58**(4): p. 354-62.
24. Piggott, L.A., et al., *The A-kinase anchoring protein Yotiao binds and regulates adenylyl cyclase in brain.* Proc Natl Acad Sci U S A, 2008. **105**(37): p. 13835-40.
25. Saucerman Jeffrey, J., et al., *Proarrhythmic Consequences of a KCNQ1 AKAP-Binding Domain Mutation.* Circulation Research, 2004. **95**(12): p. 1216-1224.
26. Nichols, C.B., et al., *Sympathetic stimulation of adult cardiomyocytes requires association of AKAP5 with a subpopulation of L-type calcium channels.* Circ Res, 2010. **107**(6): p. 747-56.
27. Carnegie, G.K., et al., *AKAP-Lbc Mobilizes a Cardiac Hypertrophy Signaling Pathway.* Molecular Cell, 2008. **32**(2): p. 169-179.
28. Fielitz, J., et al., *Requirement of protein kinase D1 for pathological cardiac remodeling.* Proceedings of the National Academy of Sciences, 2008. **105**(8): p. 3059.
29. Eide, T., et al., *Molecular Cloning, Chromosomal Localization, and Cell Cycle-Dependent Subcellular Distribution of the A-Kinase Anchoring Protein, AKAP95.* Experimental Cell Research, 1998. **238**(2): p. 305-316.
30. Huang, L.J.-s., et al., *Identification of a Novel Protein Kinase A Anchoring Protein That Binds Both Type I and Type II Regulatory Subunits.* Journal of Biological Chemistry, 1997. **272**(12): p. 8057-8064.
31. Merrill, R.A. and S. Strack, *Mitochondria: a kinase anchoring protein 1, a signaling platform for mitochondrial form and function.* The international journal of biochemistry & cell biology, 2014. **48**: p. 92-96.
32. Li, X., M.M. Nooh, and S.W. Bahouth, *Role of AKAP79/150 protein in β1-adrenergic receptor trafficking and signaling in mammalian cells.* The Journal of biological chemistry, 2013. **288**(47): p. 33797-33812.
33. Dell'Acqua, M.L., et al., *Membrane-targeting sequences on AKAP79 bind phosphatidylinositol-4, 5-bisphosphate.* EMBO J, 1998. **17**(8): p. 2246-60.
34. Kapiloff, M.S., et al., *mAKAP: an A-kinase anchoring protein targeted to the nuclear membrane of differentiated myocytes.* J Cell Sci, 1999. **112 (Pt 16)**: p. 2725-36.
35. Pare, G.C., et al., *Nesprin-1alpha contributes to the targeting of mAKAP to the cardiac myocyte nuclear envelope.* Exp Cell Res, 2005. **303**(2): p. 388-99.

36. Trotter, K.W., et al., *Alternative splicing regulates the subcellular localization of A-kinase anchoring protein 18 isoforms.* J Cell Biol, 1999. **147**(7): p. 1481-92.
37. Guillory, A.N., et al., *Enhanced cardiac function in Gravin mutant mice involves alterations in the β-adrenergic receptor signaling cascade.* PloS one, 2013. **8**(9): p. e74784-e74784.
38. Carnegie, G.K., et al., *AKAP-Lbc Nucleates a Protein Kinase D Activation Scaffold.* Molecular Cell, 2004. **15**(6): p. 889-899.
39. Taglieri, D.M., et al., *The C-terminus of the long AKAP13 isoform (AKAP-Lbc) is critical for development of compensatory cardiac hypertrophy.* Journal of molecular and cellular cardiology, 2014. **66**: p. 27-40.
40. Elbrecht, A., et al., *Molecular cloning of protein phosphatase inhibitor-1 and its expression in rat and rabbit tissues.* Journal of Biological Chemistry, 1990. **265**(23): p. 13415-13418.
41. Samuel, T.J., et al., *Correcting Calcium Dysregulation in Chronic Heart Failure Using SERCA2a Gene Therapy.* International journal of molecular sciences, 2018. **19**(4): p. 1086.
42. Kapiloff, M.S., *Contributions of protein kinase A anchoring proteins to compartmentation of cAMP signaling in the heart.* Mol Pharmacol, 2002. **62**(2): p. 193-9.
43. Hulme, J.T., et al., *A novel leucine zipper targets AKAP15 and cyclic AMP-dependent protein kinase to the C terminus of the skeletal muscle Ca2+ channel and modulates its function.* J Biol Chem, 2002. **277**(6): p. 4079-87.
44. Mauban, J.R.H., et al., *AKAP-scaffolding proteins and regulation of cardiac physiology.* Physiology (Bethesda, Md.), 2009. **24**: p. 78-87.
45. Singh, A., et al., *Analysis of AKAP7gamma Dimerization.* J Signal Transduct, 2015. **2015**: p. 371626.
46. Fung, H.Y.J., M. Birol, and E. Rhoades, *IDPs in macromolecular complexes: the roles of multivalent interactions in diverse assemblies.* Current Opinion in Structural Biology, 2018. **49**: p. 36-43.
47. Babu, M.M., et al., *Intrinsically disordered proteins: regulation and disease.* Current Opinion in Structural Biology, 2011. **21**(3): p. 432-440.
48. Ragusa, M.J., et al., *Spinophilin directs protein phosphatase 1 specificity by blocking substrate binding sites.* Nat Struct Mol Biol, 2010. **17**(4): p. 459-464.
49. Jones, D.T., K. Bryson, and L.J. McGuffin, *The PSIPRED protein structure prediction server.* Bioinformatics, 2000. **16**(4): p. 404-405.
50. Kwon, Y.G., et al., *Characterization of the interaction between DARPP-32 and protein phosphatase 1 (PP-1): DARPP-32 peptides antagonize the interaction of PP-1 with binding proteins.* Proceedings of the National Academy of Sciences of the United States of America, 1997. **94**(8): p. 3536-3541.
51. Dancheck, B., A.C. Nairn, and W. Peti, *Detailed structural characterization of unbound protein phosphatase 1 inhibitors.* Biochemistry, 2008. **47**(47): p. 12346-12356.
52. Waudby, C.A., et al., *Two-Dimensional NMR Lineshape Analysis.* Scientific Reports, 2016. **6**: p. 24826.
53. Williamson, M.P., *Using chemical shift perturbation to characterise ligand binding.* Progress in Nuclear Magnetic Resonance Spectroscopy, 2013. **73**: p. 1-16.

54. Liang, C.-T., et al., *Characterization of the interactions between inhibitor-1 and recombinant PP1 by NMR spectroscopy.* Scientific reports, 2018. **8**(1): p. 50-50.
55. Reiken, S., et al., *PKA phosphorylation activates the calcium release channel (ryanodine receptor) in skeletal muscle: defective regulation in heart failure.* The Journal of cell biology, 2003. **160**(6): p. 919-928.
56. Kimball, S.R., *Eukaryotic initiation factor eIF2.* The International Journal of Biochemistry & Cell Biology, 1999. **31**(1): p. 25-29.
57. Connor, J.H., et al., *Growth arrest and DNA damage-inducible protein GADD34 assembles a novel signaling complex containing protein phosphatase 1 and inhibitor 1.* Mol Cell Biol, 2001. **21**(20): p. 6841-50.
58. El-Armouche, A., et al., *Phospholemman-dependent regulation of the cardiac Na/K-ATPase activity is modulated by inhibitor-1 sensitive type-1 phosphatase.* The FASEB Journal, 2011. **25**(12): p. 4467-4475.
59. Picard, N., et al., *Protein phosphatase 1 inhibitor-1 deficiency reduces phosphorylation of renal NaCl cotransporter and causes arterial hypotension.* Journal of the American Society of Nephrology : JASN, 2014. **25**(3): p. 511-522.
60. Babu, G.J., M. Periasamy, and P. Bhupathy, *Regulation of sarcoplasmic reticulum Ca2+ ATPase pump expression and its relevance to cardiac muscle physiology and pathology.* Cardiovascular Research, 2007. **77**(2): p. 265-273.